Multilevel UV-B Attenuance

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Morphological and Chemical Adaptations

of *Vicia faba* to Ultraviolet-B Radiation

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geboren te Tilburg

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prof.dr. W.H.O. Ernst
prof.dr. M.A.P.A. Aerts

Contents

UV-B experiments in a greenhouse with *Vicia faba* plants. UV-B (fluorescent light) lamps and PAR (HPI-T) lamps are suspended above the plants.

Chapter 1

General Introduction

The ozone layer and solar UV-B

Without sunlight there is no life possible on earth. The sun is the ultimate energy source for ecosystems. The energy for life is delivered by the sun whereby primary producers such as green plants convert light energy into chemical energy. Also climatic processes and biogeochemical cycles are primarily driven by solar energy. In this way, the abiotic factors of an ecosystem such as radiation, rain (moisture) and temperature are directly or indirectly determined by the sun.

However, sunlight is also a potential stress factor for organisms because the solar spectrum contains harmful UV-B radiation (280-315 nm). Although the UV-B radiation is only a small part of the solar spectrum reaching the earth surface, it has a large deleterious impact on organisms because of its energizing short wavelengths (Figure 1). Moreover, biologically important molecules like proteins and nucleic acids can absorb UV-B radiation, leading to photobiological UV-B effects (Rozema *et al.* 1997, Jansen *et al.* 1998).

UV-B radiation is partly absorbed by stratospheric ozone whereas UV-A and visible light (PAR: Photosynthetically Active Radiation, 400-700 nm) are hardly absorbed by ozone. Therefore, a change in stratospheric ozone column has especially consequences for the solar UV-B levels reaching the earth surface (Figure 1). With a thinner ozone column, higher intensities of UV-B radiation and shorter wavelength radiation reach the earth surface (Madronich *et al.* 1998).

The levels of UV-B radiation reaching the earth surface show substantial temporal and spatial variation because the concentration of stratospheric ozone and the angle of the sun change in time and location above the earth surface. UV-B levels vary with longitude, altitude, albedo and clouds, time of the day and season. The dominant factor affecting UV-B radiation is the angle of the sunrays through the atmosphere. Therefore, the highest UV-B levels on earth are recorded at the equator where the solar zenith angle of the sun is smallest. Also, at noon when the solar angle is smallest, highest UV-B levels occur (McKenzie *et al.* 2003). At geological time scales, UV-B levels varied due to changing stratospheric ozone conditions and solar activity during the evolution of life on earth (Rozema *et al.* 2002).

Today's increasing UV-B levels are ascribed to ozone depletion caused by anthropogenic emission of CFCs (chlorofluorocarbons) and other ozone destroying chemicals (McKenzie *et al.* 2003). This anthropogenic increase in UV-B radiation is highest at the southern hemisphere, especially near the South Pole where an ozone "hole" occurs in spring (Farman *et al.* 1985). Also at the North Pole an increase in UV-B radiation due to anthropogenic ozone loss occurs. The losses in ozone are smaller at mid

latitudes and not significant in the tropics (UNEP 2002).

In the Montreal Protocol and its amendments (1987), many nations agreed to phase out the production and application of all CFCs, which will result in a beneficial effect on global ozone levels. However, because of the large variability in ozone level and the slow migration of the CFCs to the stratosphere, it will take several years before we can detect whether an ozone recovery occurs. A recovery of the ozone shield has been predicted for the middle of this century, on condition that emission of ozone destroying chemicals is completely stopped. However, at this moment, there are still no signs of recovery of the ozone layer and levels of UV-B radiation are still higher than 30 years ago (UNEP 2002). During the winter of 2004/2005, large-scale ozone losses of over 50 % have occurred over the Arctic (EORCU 2005). Factors like anthropogenic climate change and stratospheric polar temperatures affect the balance of ozone production and breakdown, which makes it complex to predict the recovery of the ozone layer (Shindell *et al.* 1998). Thus, although the emission of ozone destroying chemicals is reduced, the ozone layer and enhanced levels of UV-B on earth are still a major concern.

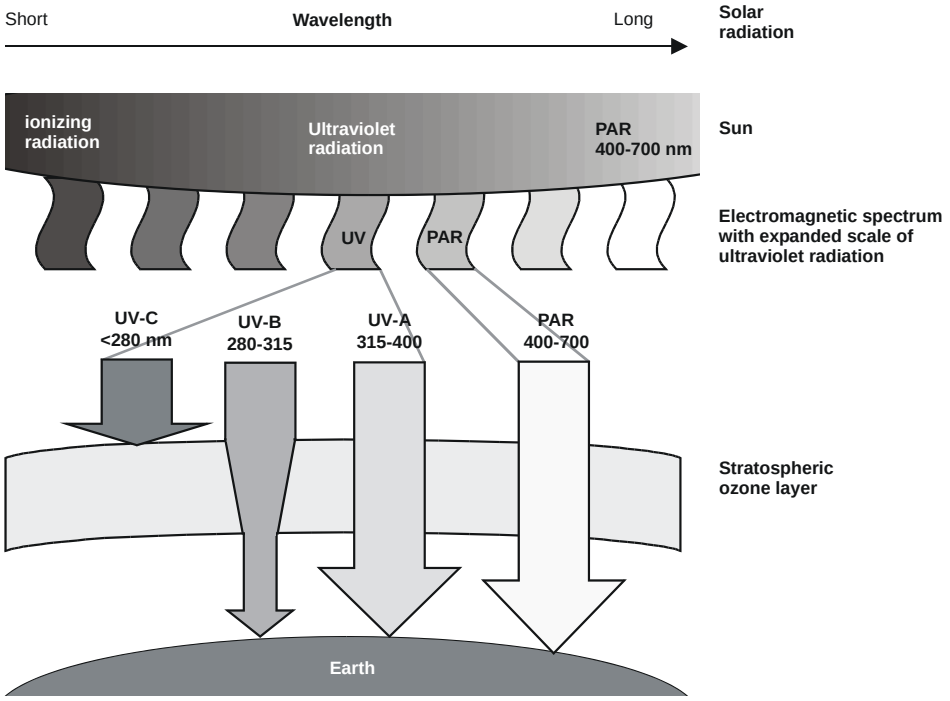

Figure 1. The electromagnetic radiation emitted by the sun is composed of radiation with different wavelength (nm) of which a small part is ultraviolet (UV). UV-C radiation is completely absorbed by stratospheric ozone, UV-B partly and UV-A not, so that only wavelength radiation of > 290 nm reaches the earth surface.

UV-B effects on plants: from molecular targets to the whole plant level

Balance between damage and adaptation

Micro-scale differences in UV-B doses occur between habitats so that organisms experience different UV-B doses and are adapted to UV-B levels to a different extent. The necessity for plants to cope with the harmful UV-B radiation is higher in open habitats where relatively high UV-B levels reach the soil surface (e.g. dune grassland, alpine grassland) than in habitats with a much denser canopy (e.g. forest underground) (Frohnmeijer & Staiger 2003). Consequently, adaptation and damage processes of plant species also differ in response to anthropogenically enhanced UV-B radiation. Even within one plant species, differences in plant responses occur, especially when they occupy different habitats (Rozema *et al.* 1997, Jansen *et al.* 1998, Kakani *et al.* 2003).

Especially at high UV-B radiation levels (comparable to more than 20 % ozone reduction) plant growth is reduced in a lot of species including crop species (Kakani *et al.* 2003, Caldwell *et al.* 2003). Adapted plants will show no visible UV-B damage such as plant growth reduction but can exhibit more stunted plant architecture and altered leaf morphology, which provide more attenuation of UV-B. Also altered leaf chemistry with higher flavonoid, tannin and lignin contents leads to increased UV-B attenuation (Rozema *et al.* 1997).

Plant performance in response to enhanced UV-B radiation is the balance between cellular damaging and repair processes on one hand and adaptation mechanisms after UV-B exposure on the other hand. Therefore, to assess the total UV-B sensitivity and tolerance of plant species and cultivars, the growth of the whole plant is considered to be a good parameter (Jansen *et al.* 1998, Frohnmeijer & Staiger 2003, Brosché & Strid 2003). The primary molecular targets are the bases for the damaging and adaptive UV-B effects at leaf and plant level. Harmful effects of the high-energy UV-B radiation can be caused by photodestruction of biomolecules like DNA. When molecular processes in the cells are triggered and regulated directly or indirectly by UV-B light, repair and/or adaptation processes like flavonoid induction also occur. The balance between these deleterious and UV-B regulated cell processes determines the UV-B effects at plant level: the plant performance (Jansen *et al.* 1998).

Damaging effects

High doses of UV-B are damaging cellular components. The energy of the radiation is that high that it causes photochemical changes to important biomolecules such as proteins and nucleic acids (Jansen *et al.* 1998, Greenberg *et al.* 1997). High levels of UV-B can also induce the reactive singlet state oxygen molecule ($^1O_2$), which can lead to tissue damage (Greenberg *et al.* 1997). The photodestruction of biomolecules leads to several direct UV-B effects like DNA damage (see box 1). Specific cellular UV-B receptors are not involved in these reactions. This damage can result in general stress responses such as wound signalling or repair mechanisms, reduced plant growth or decreased fecundity which are indirect regulated effects (Brosché & Strid 2003, Frohnmeijer & Staiger 2003, Jansen 2002).

DNA is one of these molecular targets, which is very sensitive to UV-B radiation with far reaching consequences. The major damage is the formation of CPDs (cyclobutane pyrimidine dimers) and (6-4') photoproducts (Jordan 1996). When the damage is not repaired, gene transcription and DNA replication are blocked. This results in mutations, a disturbed cell cycle and disrupture of cellular metabolism (Box 1, Brosché & Strid 2003, Jansen *et al.* 1998, Jordan 1996).

Proteins absorb UV-B radiation and are therefore UV-B sensitive. Because of the ubiquitous function of proteins in the cell it can affect nearly all cell processes, metabolism and cell structures. For instance the main enzyme in the Calvin cycle (Rubisco) may be inactivated by UV-B. In this way, photosynthesis is reduced (Greenberg *et al.* 1997). However, Rubisco is not a unique target for UV-B in the photosynthetic machinery. Also photosystem II, the thylakoid membrane, chlorophyll and carotenoids can be damaged or inactivated so that the photosynthesis is reduced (see Box 1).

Other molecular targets are biomembranes. Especially the polyunsaturated fatty acids are susceptible to oxidative damage. Peroxidation of lipids can take place so that the biomembrane function and permeability are disturbed. Also ATPases in the membrane can be inhibited by UV-B (Greenberg *et al.* 1997).

Finally, phytohormones, especially indoleacetic acid (IAA) are targets for UV-B (see Box 1). IAA is important for cell elongation and apical dominance. Therefore, altered IAA levels have consequences for plant morphology (Jansen 2002). Concentrations of IAA can be reduced directly via photooxidation but also indirectly, by binding of IAA to flavonoids. It is still not elucidated if the IAA levels are regulated via a specific UV-B receptor and/or if the levels are a result of photochemical reactions. Therefore plant alterations, regulated by IAA could be a specific UV-B adaptive response or could be an unforeseen effect, which occurs after damage to IAA (Jansen 2002).

Box 1. Plant responses to UV-B: damage and adaptation reactions

Class of biomolecules	**Specific molecular target**	**Cellular damaging effects and damaged processes**	**Main adaptation mechanisms**	**Protective mechanisms**
Nucleic acids	DNA	Mutations Cell cycle and cell disrupture Gene transcription	DNA repair	Photoreactivation Excision repair
Proteins	Rubisco Photo System II ATPases Other enzymes	Photosynthesis Photosynthesis Membrane transport Metabolism, general cell functioning	Reactive oxygen species (ROS) scavengers	Antioxidants (e.g. carotenoids, gluthathione, α tocopherol, phenolics) Antioxidant enzymes (e.g. superoxide dismutase)
Lipids	Unsaturated fatty acids in membranes	Membrane permeability. Thylakoid membrane: photosynthesis	UV-B absorbing screen in epidermis	Increase in flavonoids and hydroxy cinnamic acids
			Changed plant chemistry	Lignin and tannin content Polyamines in the membranes
Pigments	Chlorophyll	Photosynthesis	Scattering and reflection of UV-B	Altered wax layer Altered epidermal and cuticular structures Other leaf optical properties.
Phytohormones	indoleacetic acid (IAA)	Cell elongation and cell growth	Avoid UV-B irradiation by altered morphology	Thicker leaves More branching Shorter plant height Smaller leaves and leaf area Leaf curling
	Whole plant performance :	Plant growth Plant architecture Fecundity and Reproduction Competitive balance		

Adaptations to UV-B at the plant and tissue level

Since plants lack mobility, they cannot escape UV-B stress. Therefore, they possess several adaptation and repair mechanisms to cope with (enhanced) UV-B radiation. A lot of definitions for adaptation are used. In this thesis, the following meaning of adaptation is used: "A genetically determined characteristic (behavioral, morphological, or physiological) that improves an organism's ability to survive and reproduce under prevailing environmental conditions" (Smith & Smith).

Adaptation to UV-B generally consists of responses like an altered plant growth, plant architecture and leaf morphology, enhanced flavonoid biosynthesis, DNA repair and enhanced reactive oxygen species (ROS) scavenging capacity (see Box 1). These indirect UV-B effects are subtle and triggered at low UV-B fluency rates that are too low for photochemical degradation of the biomolecules (Jansen *et al.* 1998). In contrast, direct UV-B effects are caused by the energizing UV-B radiation without interference of a regulation mechanism.

Plants can adapt to UV-B radiation by attenuating UV-B before this radiation can be harmful in the photosynthetic cells and the nucleus. This attenuance is possible at the whole plant level by a more stunted morphology such as shorter plants and internodes with increased branching (Box 1) (Krizek 2004, Day & Neale 2002).

The reduction of deleterious UV-B radiation also occurs in leaf tissues. In the leaf five factors (1-5) generally determine the UV-B attenuance. The first three factors (1. molar absorptivity, 2. concentration of UV-B absorbing compounds, 3. pathlength) are components of Beer's law for absorption in a solution:

$A = \varepsilon * l * c$. (eq. I)

$A = -\log T$. (eq. II)

$T = I_0 / I$. (eq. III)

A: absorption, ε: molar absorptivity (L mol^{-1} cm^{-1}), l: pathlength of the light through a solution (cm), c: molar concentration of the absorbing compounds (mol L^{-1}). T: transmission, I_0: initial light intensity, I: light intensity after transmission through a solution.

Thus the attenuance of UV-B in a leaf is determined by:

1. Molar absorptivity. Some specific types of molecules like flavonoids and hydroxy cinnamic acids have good UV-B absorbing properties and are suitable as UV-B absorbing compounds in the leaf. This property is reflected in the molar absorptivity (ε) of formula I. The flavonoids form a UV-B absorbing screen in the epidermal layer of the leaf,

which protects the underlying photosynthetic tissues. PAR is not absorbed by these compounds and can reach the mesophyll unattenuated (chapter 2 of this thesis, Krizek 2004).

2. High concentrations of UV-B absorbing compounds ("c" in formula I). There is a logarithmic relation between concentration and percentage of light transmitted (formula II and III). The concentration of phenolics is increased in response to UV-B in a lot of plant species so that the UV-B attenuance is enhanced (chapter 2 of this thesis, Markstädter *et al.* 2001).

3. Pathlength of light. "l" in formula I is determined by morphological factors like leaf thickness, wax layers and scattering, which is dependent on cell and tissue structures. This factor can be estimated by measuring the UV attenuance of leaves of which all the extractable components have been removed. Specific leaf weight is a measure for leaf thickness and also gives an indication of the pathlength of the light. (Barnes *et al.* 2000).

4. An effective localisation of the UV-B absorbing compounds contributing to an optimal screening function. A heterogeneous distribution may enhance the effectiveness if the flavonoids are located on the adaxial side, on top of the mesophyll. On the other hand, a non-homogeneous distribution, whereby gaps in the UV screen occur, reduces the effectiveness (Markstädter *et al.* 2001).

5. Reflection of UV-B light via the cuticle. In some plants reflection plays a major role in UV-B protection, whereas in other plants (such as *V. faba*) reflection is a minor factor in attenuation by leaves (González *et al.* 1996).

In addition to the screening of UV-B radiation, flavonoids play probably also a role as antioxidative agent to scavenge ROS. Also various other antioxidants and stress-induced enzymes are synthesised in response to UV-B (Jordan 1996). To protect biomembranes, enhanced amounts of polyamines are produced, especially at high PAR levels, which could enhance the stability of the biomembrane so that UV-B damage is restricted (Jordan 1996) (see Box 1). To repair DNA, plants are equipped with several repair mechanisms. During photoreactivation, monomerization of the dimers by photolyases takes place. These enzymes are activated by high PAR levels, partly explaining the mitigating effect of high PAR levels on UV-B damage (Day & Neale 2002). With excision repair mechanisms, the damaged part of the DNA is removed and replaced by newly replicated DNA (Britt 1996).

Vicia faba as a model system

In this thesis, *Vicia faba* L. (Faba bean) is used as a model system to explore the effects and adaptations of plants to UV-B radiation. This species has been chosen for several reasons:

1. *V. faba* is a crop plant of agricultural importance. There are many cultivars available with different characteristics so that a sensitive and less sensitive cultivar could be selected (Biggs *et al.* 1981). Moreover, this crop plant has a very homogeneous development and growth.
2. The evolutionary and biogeographical origin of *V. faba* is situated around the Mediterranean Sea and Western Asia, regions with variable and high levels of solar UV-B radiation. Therefore it is expected that this plant species has evolved adaptation mechanisms to solar UV-B radiation.
3. *V. faba* has a compound leaf consisting of different leaflets that are easy to sample for measurements of different parameters. In addition, leaves will be similarly exposed to UV-B doses since the planophyllic leaves generally have a horizontal position.
4. Preliminary results showed that *V. faba* is a relatively UV-B sensitive plant species (Tosserams *et al.* 2001).

Plant performance of different cultivars of *V. faba*

Differences in UV-B damage and adaptation to UV-B are to be expected, even within cultivars of one crop species. Therefore, the research was started with screening five (commercially available) cultivars of *V. faba*, differing in sensitivity to UV-B radiation. To determine the UV-B tolerance, six parameters were determined which reflect UV-B damaging and adaptation responses. The percentage of decrease or increase in response to UV-B was estimated. Details about the methodology are presented in Box 2.

For each parameter, the cultivars were ranked according to sensitivity to elevated UV-B. Higher sensitivity corresponds with a higher rank number. The Relative Growth Rate (RGR), Net Assimilation Rate (NAR), plant height, leaf area, Specific Leaf Area (SLA) and UV-B absorbing compounds were the parameters contributing to the final sensitivity ranking (Biggs *et al.* 1981). Calculations to estimate these parameters are indicated in Box 2. The rank numbers for these six parameters were summed up per cultivar, which resulted in a total ranking for UV-B sensitivity or tolerance (Table 1).

Overall, the number of significant UV-B responses was limited. The concentration of UV-B absorbing compounds in the leaves was the most responsive parameter. The UV-B absorbing compounds were significantly increased in response to UV-B in nearly all

Box 2. Methodology for screening the sensitivity of the *V. faba* cultivars

Greenhouse experiment	PAR: > 250 μE m^{-2} day^{-1} (canopy height) Start treatment 14 days after sowing. UV-B treatment: 26 days. Treatments "square wave" irradiation (Day & Neale 2002): 1. *plus* UV-B (cellulose acetate foil; UV-B $_{BE}$: 12 KJ m^{-2} day^{-1} weighted with the general plant action spectrum (Caldwell 1971)). 2. *minus* UV-B (Mylar foil). For further details about growth conditions and light treatments, see: Chapter 3 of this thesis
Parameters:	Total UV-B absorbing compounds: relative absorbance ($_{\lambda 280}\int^{\lambda 320}$) in 80 % methanol per leaf area (absorbance/ cm^2). Relative Growth Rate: $RGR = \frac{\ln W_2 - \ln W_1}{t_2 - t_1}$ (eq. IV) W_1 and W_2: Total plant biomass (g dry weight) at t_1 : start of the growth experiment and t_2: end of the growth experiment (28 days). Net Assimilation Rate: $NAR = \frac{\ln A_2 - \ln A_1}{t_2 - t_1} * \frac{W_2 - W_1}{A_2 - A_1}$ (g m^{-2} day^{-1}) (eq. V). A_1 and A_2: Total leaf area (m^2) at t_1 and t_2: (28 days). Specific Leaf Area $SLA = \frac{A_2}{W_{leaf}}$ (m^2 g^{-1}) (eq. VI). W_{leaf}: leaf biomass (g dry weight) Plant height (m), leaf area (m^2) Number of shoots and leaves (not used for evaluating the sensitivity) For details: see further: Chapter 3 of this thesis Statistics: One Way ANOVA
V. faba Cultivars:	Caspar (CEBECO Zaden BV, Lelystad, NL) Bartiny (Barenbrug, Wolfheze, NL) Pistache (Joordens, Neer, NL) Condor (NPZ Hohenlieth, D) Minica (Nickerson-Zwaan, Barendrecht, NL)

cultivars (except cv. Caspar) (Table 1). This observation supports the hypothesis that the leaf chemistry in UV-B attenuation (type and concentration of the compound) is important for the protection of plant tissue to UV-B.

The Relative Growth Rate (RGR) is regarded a good measure to assess the overall UV-B effects for individual plants since it integrates Net Assimilation Rate (NAR), potential photosynthetic leaf area and Specific Leaf Area (SLA). Cv. Pistache showed a significant decrease in RGR whereas the other cultivars did not (Table 1). No significant decrease in NAR was observed in the five tested *V. faba* cultivars (Table 1), which indicates that photosynthesis was not severely damaged by UV-B. This result is also confirmed by research of Tosserams *et al.* (2001). Also the other parameters, which influence the growth, e.g. SLA and total leaf area, are not significantly altered in

Table 1. Ranking of the UV-B responses (as a percentage of the control, between parentheses) on RGR, NAR, leaf area, SLA, plant height and UV-B absorbing compounds, number of leaves and shoots of 5 *V. faba* cultivars. High rank number means high sensitivity.
Significant UV-B responses per cultivar are indicated: *: p<0.05, **: p<0.01, *** p<0.001 (One Way ANOVA).

Ranking number	RGR	NAR	Leaf area	SLA	Plant height	UV-B absorbing com-pounds	Number of leaves	Number of shoots	Final ranking [ranking points]
1 tolerant	Caspar (102.1)	Minica (99.8)	Minica (105.0)	Minica (94.2)	Caspar (92.1)*	Minica (136.7)**	Minica (128.5)**	Minica (138.9)**	Minica [9]
2	Minica (101.2)	Caspar (98.4)	Caspar (95.7)	Bartiny (94.6)	Bartiny (88.7)	Bartiny (125.7)***	Condor (103.3)	Condor (111.1)	Caspar [13]
3	Condor (96.0)	Condor (96.5)	Condor (88.6)	Caspar (95.7)	Minica (88.7)**	Condor (119.7)**	Bartiny (103.3)	Pistache (96.3)	Bartiny [20]
4	Bartiny (94.0)	Pistache (94.9)	Pistache (88.1)	Condor (100.1)	Condor (88.2)	Caspar (115.0)	Caspar (92.9)	Caspar (92.3)	Condor [20]
5 sensi-tive	Pistache (89.5)**	Bartiny (94.6)	Bartiny (87.0)	Pistache (100.3)	Pistache (78.9)***	Pistache (109.4)**	Pistache (86.1)*	Bartiny (91.4)	Pistache [28]

response to UV-B in all tested cultivars. SLA values in Table 1 were based on total leaf biomass and leaf area calculations. However, Specific Leaf Weight ($SLW=SLA^{-1}$) per individual leaf would be a more precise method to estimate leaf thickness (B. Meijkamp, unpublished results).

The parameters affecting UV-B attenuance and photomorphogenesis (i.e. plant height, UV-B absorbing compounds, number of leaves and shoots), which reflect UV-B adaptation effects, showed more pronounced differences between cultivars (Table 1). The plant height was significantly reduced in response to UV-B radiation in three cultivars (see Table 1). Concomitant with the significant increase in number of leaves in cv. Minica the number of shoots increased significantly. This indicates that the whole plant morphology of cv. Minica changed (i.e. more branched plants) in response to UV-B. The number of leaves was reduced in cv. Pistache.

Also leaf morphology was altered in response to UV-B so that high UV-B radiation levels could be reduced in leaf tissue. Although no significant UV-B response in leaf area was observed after 26 days of UV-B irradiation (Table 1), the size of the individual leaves was significantly decreased in response to UV-B in both cultivars Minica and Pistache

(chapter 3 of this thesis). Leaf thickness of mature leaves of these two *V. faba* cultivars were significantly increased in response to UV-B (chapter 4 of this thesis).

The final ranking based on a combination of adaptation and damaging parameters suggests that cv. Minica is less sensitive to UV-B than cv. Pistache (Table 1). However, the significant UV-B effects of the parameters, which could show damaging UV-B growth responses (RGR, NAR, leaf area, SLA), were limited.

Nevertheless, subtle UV-B effects on morphology and chemistry of individual plants could have far reaching consequences for the plant and an ecosystem as a whole. The consequences of enhanced UV-B radiation for intensively managed ecosystems such as crop fields are predicted in several assessments of effects of the depletion of the ozone layer (UNEP 2002). For crops like *V. faba,* the growth could influence the final yield. Moreover plant architecture influences plant density and thus indirectly the competitive balance and yield. Furthermore an altered chemistry may affect food quality, taste and the degree of herbivory. In addition, alterations in fertility may affect the final yield, for instance when the seeds are harvested as agricultural product (Kakani *et al.* 2003, Caldwell *et al.* 1995).

1.4 Aims and outline of this thesis

The main aim of this thesis was to study plant responses and adaptation mechanisms to enhanced UV-B radiation at various hierarchical levels (whole plant, leaf tissue, cell). Two cultivars *of V. faba*, cv. Pistache and Minica, differing in UV-B sensitivity, were used as a model system.

It was hypothesised that secondary metabolites, especially the phenolics are crucial in UV-B protection because of their UV-B absorbing properties (Rozema *et al.* 1997, Table 1). Therefore, the main part of this thesis was dedicated to the UV-screening function of flavonoids in the leaves. However, a comprehensive overview of the secondary metabolites in plants in response to UV-B was absent. This gap was filled with a literature review (**Chapter 2**) addressing the following questions:

- What are the main secondary plant metabolites, their metabolic pathways and the methods to determine the levels of these compounds that are involved in UV-B absorption in different plant groups?
- Is the function of these compounds exclusively restricted to UV-B absorption?
- Which environmental factors determine the level of the UV-B absorbing compounds in plant tissues?

The experimental studies, described in this thesis (Chapter 3, 4 and 5) were performed in a greenhouse with lower PAR levels than outdoor studies. High PAR levels

enhance photorepair mechanisms so that less DNA damage occurs. Moreover, PAR levels could modify the photomorphogenic responses (Rozema *et al.* 1997, Day & Neale 2002, Krizek 2004). It was hypothesised that lower PAR levels in a greenhouse intensify the UV-B responses and increase sensitivity in several ways. The effects of low and near-ambient PAR levels on the UV-B responses in *V. faba* are assessed in **chapter 3**. The main research question was:

- What are the UV-B effects of *V. faba* on growth, morphology and UV-B absorbing compounds with low and near-ambient PAR levels?

UV-B screening by forming a screen of flavonoids is an important adaptation to UV-B in *V. faba* plants (Markstädter *et al.* 2001, Barnes *et al.* 2000). According to Beer's law, concentration, type of flavonoids and leaf thickness determine the UV attenuance. Since UV-A can also induce pigment formation (Beggs & Wellmann 1994), the effect of increased UV-A levels were studied as well. These factors are addressed in **chapter 4** with the following research questions:

- What are the levels of *in vivo* UV attenuance in *V. faba* leaves in different developmental leaf stages in response to UV-A and UV-B radiation?
- What is the contribution of leaf thickness, non-soluble phenolic compounds and flavonoids to UV attenuance?
- Is there a specific induction of flavonoids in response to UV-A and UV-B radiation?
- Is there a difference in UV-B attenuation between two cultivars of *V. faba* (cv. Pistache and Minica), varying in sensitivity to UV-B?

The screening effectiveness of flavonoids is also determined by the tissue specific localisation of these flavonoids within the leaf tissues (chapter 2 of this thesis). This was addressed in **chapter 5** with the next question:

- What is the localisation of constitutive and UV-B induced kaempferol and quercetin flavonoids in *V. faba* leaf tissues?

Chapter 6 is a concluding chapter, summarising the results of this thesis.

Faba bean (*Vicia faba*) synthesises higher amounts of flavonoids in response to UV-B.

Chapter 2

Effects of UV-B on secondary metabolites in plants

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Wilfried H.O. Ernst, Jelte Rozema

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Abstract

Increasing levels of UV-B radiation reaching the earth affect the secondary metabolism of plants. Especially the phenylpropanoid pathway is influenced by UV-B. Products of this pathway like flavonoids and hydroxycinnamic acids have favourable UV-B absorbing properties and are thus very effective as UV-B protective pigments in plants. An overview of analytical methods to determine nature and concentration of these UV-B absorbing compounds in plants is given.

There is a high variability in quality and quantity of phenolics in plants depending on life form, species and cultivar, and localisation within plant, tissue and cell. Also developmental stage of the plant, time of the day, and UV-B dose are responsible for differences in the amount and kind of phenolics.

Besides the absorbing properties, other properties of phenolics are also discussed. For example, the interaction between UV-B absorbing pigments and growth regulators influences the morphology of plants and can in this way be adaptive for plants growing in a high UV-B environment.

Introduction

During the last decades the stratospheric ozone layer is decreasing in thickness, due to increased emission of reactive anthropogenic organic compounds such as chlorinated fluorocarbons (CFCs), which break down ozone (Herman *et al.* 1996). Besides global warming at the earth's surface, greenhouse gases also lead to cooling of the stratosphere, which increase the arctic ozone hole (Shindell *et al.* 1998). Because ozone partly absorbs UV-B, levels of UV-B radiation on earth are increasing when the ozone layer is broken down (Herman *et al.* 1996). Thus anthropogenic pollution elevates the natural UV-B dose to which plants are exposed.

High UV-B fluxes are potential harmful to plants. Damage can occur to DNA by formation of DNA dimers. UV-B can also destroy parts of the photosynthetic apparatus (especially photosystem II), proteins and membranes (Jansen *et al.* 1998, Tevini & Teramura 1989). Because terrestrial plants are exposed to high levels of UV-B radiation in comparison to aquatic plants, it is likely that they have developed adaptation mechanisms to prevent UV-B damage (Rozema 1999, Cooper-Driver & Bhattacharya 1998, Rozema *et al.* 1998, Stafford 1991). One of the adaptation mechanisms is the enhanced production of secondary metabolites in leaves, which absorb UV-B radiation (Bornman *et al.* 1997, Cooper-Driver & Bhattacharya 1998, Dixon & Paiva 1995). The accumulation of secondary metabolites thus prevents damage to DNA, membranes,

proteins and photosynthetic tissues by lowering UV-B levels within the tissues. Oxidative damage is decreased by the reactive oxygen species (ROS) scavenging properties of these phenolics (Beggs & Wellmann 1994, Cooper-Driver & Bhattacharya 1998, Day *et al.* 1992, Jansen *et al.* 1998, Landry *et al.* 1995, Stapleton & Walbot 1994, Tevini *et al.* 1991). Probably, these compounds can also directly or indirectly regulate morphogenetic changes such as thickening of leaves, which also reduces the UV-B dose in the photosynthetic tissues (Jansen *et al.* 1998, Lois 1994, Stafford 1991). Many studies showed elevated concentrations of flavonoids in response to UV-B. Table 1 gives evidence that enhanced levels of phenolics such as flavonoids are present in the leaves in response to UV-B. Apart from flavonoids, simple phenolics and hydroxycinnamic esters also seem to be important in absorbing UV-B radiation (Bornman *et al.* 1997, Caasi-Lit *et al.* 1997, Dixon & Paiva 1995, Fischbach *et al.* 1999, Krauss *et al.* 1997, Landry *et al.* 1995, Liu *et al.* 1995, Reuber *et al.* 1996b, Sheahan 1996). However, only limited experimental data are available on these compounds (Table 2).

The aim of this chapter is to give an overview of the secondary metabolites, which are involved in the absorption of UV radiation. It seems that the induction of the phenylpropanoid pathway is a universal adaptation mechanism to UV-B radiation in the plant kingdom (Beggs & Wellmann 1994, Bornman *et al.* 1997). For this reason, we first give a summary of the shikimate and phenylpropanoid pathway. Then, we highlight the compounds of these pathways, which are induced by UV-B. Next we compare the different methods to quantify the UV-B absorbing pigments. Further, an inventory was made of quantity and quality in the UV-B induced compounds of the phenylpropanoid pathway in plants and potential implications are discussed. Because the function of the UV-B absorbing pigments is probably not restricted to UV-B screening, other physiological and ecological functions of these compounds will be discussed in the last section.

Pathways of important UV-B absorbing compounds.

UV-B induced compounds are often phenolics. The synthesis of phenolics in plants occurs via the shikimate and phenylpropanoid pathway. The shikimate pathway links the sugar metabolism with the phenylpropanoid metabolism. The synthesised carbohydrates are directed towards the phenolic metabolism instead of being used in the primary metabolism. The end product of the shikimate pathway is the amino acid phenylalanine (Figure 1). The phenylpropanoid pathway starts with cinnamate (Figure 2). Depending on environmental stress factors, through this pathway one or more related phenolic

compounds can be produced in enhanced amounts (Beggs & Wellmann 1994, Dixon & Paiva 1995, Harborne 1989)

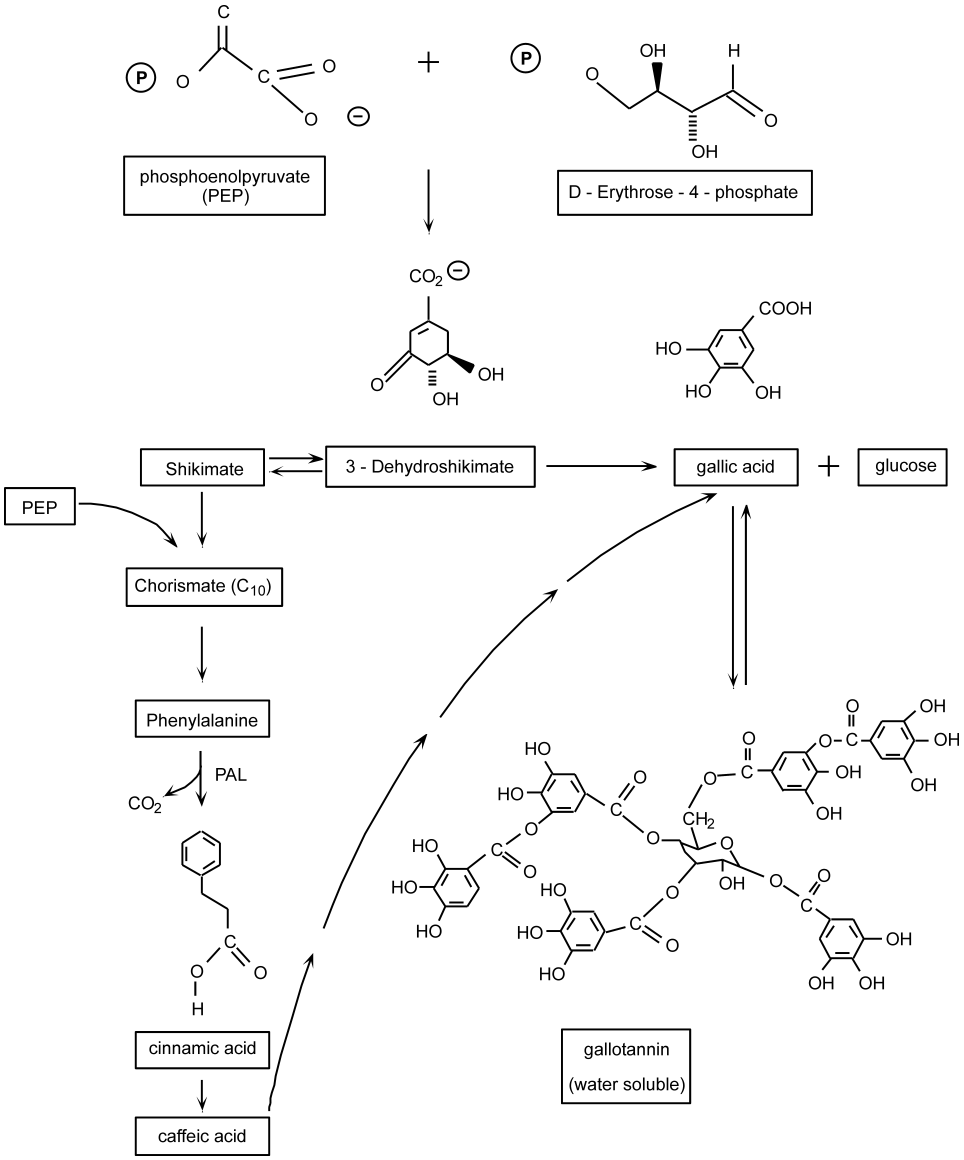

Figure 1. The shikimate pathway (Based on Haslam 1981).

Shikimate pathway.

Figure 1 shows that shikimate has a key position in the metabolism of aromatic compounds. The precursors of the shikimate pathway that originate from the sugar metabolism are D-erythrose-4-phosphate (C_4) and phosphoenolpyruvate (PEP)(C_3) (Figure 1). Erythrose-4-phosphate is synthesized in the pentose phosphate pathway and phosphoenolpyruvate is formed during glycolysis. Via some intermediates, 3-dehydroshikimate and shikimate are synthesized from erythrose-4-phosphate and phosphoenolpyruvate. A second PEP molecule is linked to shikimate, which gives chorismate (C_{10}). Starting from chorismate the amino acid phenylalanine is synthesized (Figure 1). Phenylalanine then can be used in the phenylpropanoid pathway (Figure 2). From 3-dehydroshikimate, also gallic acid can be derived (Figure 1). Gallic acid, combined with glucose, forms the building block for the hydrolysable gallotannins. As can be seen in Figure 1, more pathways lead to the formation of gallic acid. For example caffeic acid is synthesized from phenylalanine. From caffeic acid, via some intermediates, gallic acid can also be formed (Harborne 1980, Haslam 1981).

Phenylpropanoid pathway.

The phenylpropanoid pathway starts with phenylalanine and ends with different (complex) phenolics. Figure 2 shows a scheme of the phenylpropanoid pathway (modified from Dixon & Paiva 1995, and Beggs & Wellmann 1994). Phenylalanine is condensed to cinnamate by the key enzyme PAL (phenylalanine ammonium lyase). Via some intermediates salicylic acid is formed. *p*-Coumarate can be synthesized from both cinnamate and tyrosine (Dixon & Paiva 1995, Grisebach 1981, Rice-Evans *et al.* 1997). The activated form of *p*-coumarate is the branch point for the pathways leading to the synthesis of flavonoids, pterocarpans, stilbenes, furocoumarins and hydroxycinnamic acids like caffeic acid, ferulic acid (=coniferyl acid) and sinapic acid, which are the precursors for lignin (Figure 2) (Beggs & Wellmann 1994, Chapple *et al.* 1992, Dixon & Paiva 1995, Dixon *et al.* 1996, Fritzemeier & Kindl 1981, Grisebach 1981, Harborne 1980, Shirley 1996).

For lignin synthesis, hydroxycinnamic acids can be reduced to alcohols: *p*-coumaric acid to *p*-coumaryl alcohol, sinapic acid to sinapyl alcohol and ferulic acid to coniferyl alcohol by converting the acid group into a hydroxyl group (Grisebach 1981). Lignin of dicotyledons is formed by dehydrogenation of these three alcohols (*p*-coumaryl alcohol, sinapyl alcohol and coniferyl alcohol), whereas lignin of gymnosperms mainly consists of coniferyl alcohol (Grisebach 1981).

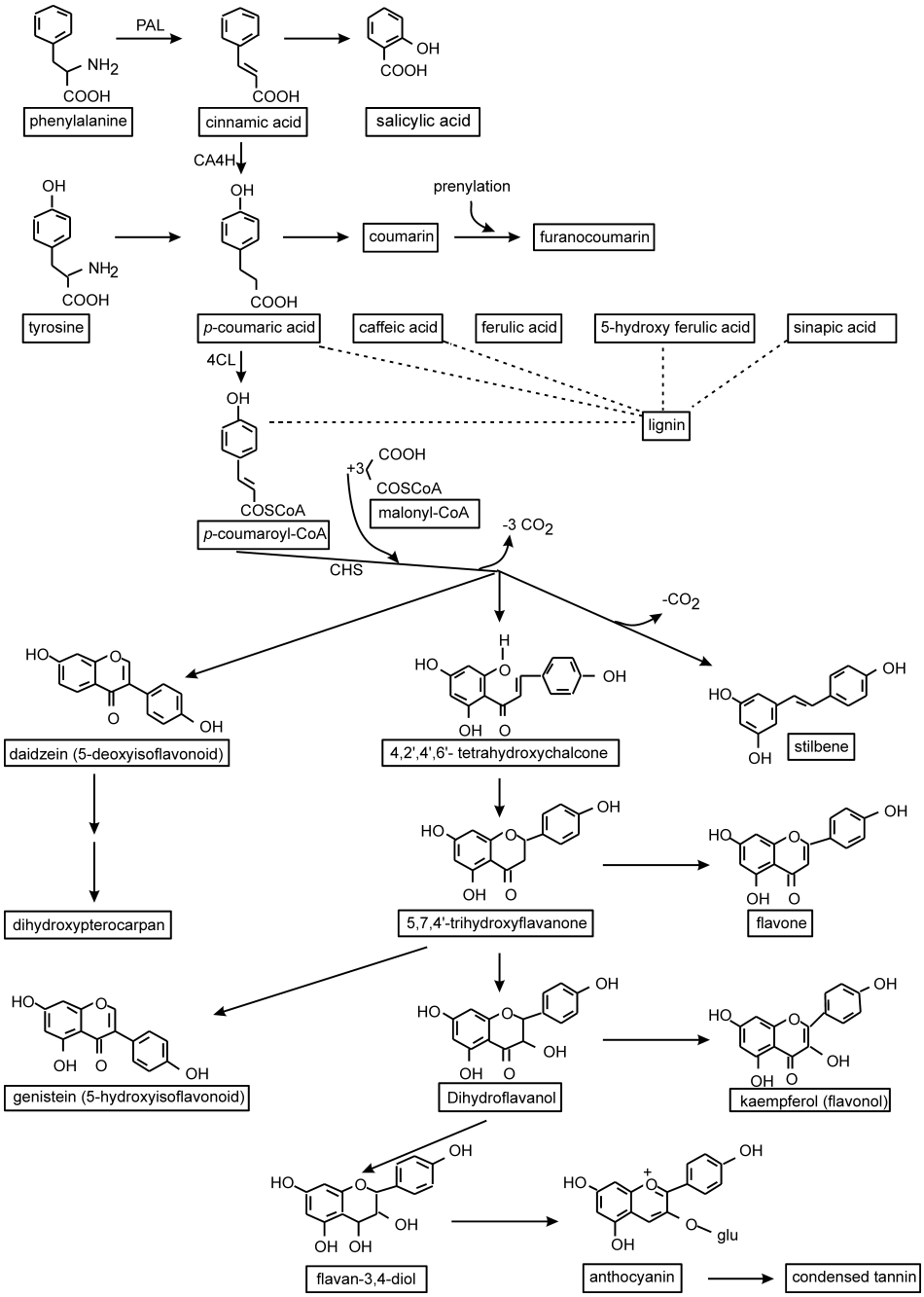

Figure 2. Overview of the phenylpropanoid pathway (modified after Dixon & Paiva 1995).

The phenylpropanoid pathway, together with the polyketide pathway, is the starting point for flavonoids and stilbenes (Beggs & Wellmann 1994, Grisebach 1981, Harborne 1980, Rice-Evans *et al.* 1997) (Figure 2). For flavonoids, *p*-coumaroyl-CoA is combined with three malonyl units and three molecules CO_2 are removed. First a chalcone is formed, which can be condensed to flavanones, flavanols, flavones, flavonols, flavanes and anthocyanins (see Figure 2) (Beggs & Wellmann 1994, Hahlbrock 1981, Harborne 1989, Rice-Evans *et al.* 1997).

By transferring the B-ring from the 2 to the 3 site of the C-ring (Figure 3), an isoflavonoid is synthesized (Figure 2). Pterocarpans, isoflavones and isoflavanones are different groups of isoflavonoids (Figure 2) (Beggs & Wellmann 1994).

When the base structure of the flavonoid (the aglycone) is formed, in many cases it will be glycosylated or polymerized (Beggs & Wellmann 1994, Harborne 1980). Polymerization of anthocyanidins gives the non-hydrolysable tannins. Both reactions will be discussed below.

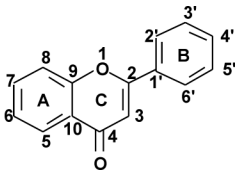

Figure 3. The flavonoid skeleton structure (Modified after Markham 1982).

Glycosylation and polymerization of phenolics to flavonoid glycosides and tannins.

Most flavonoids and phenolic acids are not accumulated in free or aglycone form in the leaves. They mostly conjugate with sugars, cell wall carbohydrates (e.g. ferulate esters) and organic acids (e.g. sinapate esters, chlorogenic acid) (Dixon & Paiva 1995, Harborne 1980). Also conjugation of flavonoids with phenyl propanols, isoprene, methyl and sulphate is found in plants (Charest *et al.* 1986, Grandmaison & Ibrahim 1996, Harborne 1989, Harborne 1980). However, aglycones are found in leaves too, for example in the wax layer of leaves (Stephanou & Manetas 1997, Wollenweber & Dietz 1981)

A last step in the flavonoid synthesis can be the glycosylation of the flavonoids (Beggs & Wellmann 1994, Harborne 1980). Glycosylated flavonoids are called (flavonoid) glycosides. The advantages of glycosylation of flavonoids are the improved water solubility and the higher chemical stability of the glycosides. Also the lower toxicity of the glycosides is of biological importance. These characteristics of glycosides make them more suitable for cell metabolism than the aglycone flavonoid. Most of the glycosylation of flavonoids (as well as other secondary metabolites) are catalysed by a glycosyl

transferase, which transfers the sugar of a nucleotide-activated sugar to the flavonoid. This hydrolysis is in most cases exothermic (Harborne 1980, Hösel 1981). The glycosyl transferases can be divided into two types: those that bind a sugar to the hydroxyl group of a flavonoid and those that bind a sugar to a hydroxyl group of a sugar, that is already linked to the aglycone. Monosaccharides such as glucose, galactose, rhamnose, xylose and arabinose are the sugar compound in the glycoside. However, disaccharides such as rutose are also found (Harborne 1980, Harborne 1989, Rice-Evans *et al.* 1997).

Another reaction type, which can take place after the formation of the flavonoid skeleton, is a polymerization to tannins. The non-hydrolysable tannins or proanthocyanidins are polymers of flavan-3-ol. An example of such a flavan-3-ol is catechin. In most cases the C-4 is linked to the C-8 or C-6 (Dixon & Paiva 1995, Haslam 1981).

Table 1. Percentage increase of flavonoid concentrations in response to UV-B (control: 100 %). In brackets: absolute concentrations for UV-B treatment.
Experimental set-up: Experiment carried out in growth chamber (C), greenhouse (G) or field filter experiment(F); UV-B doses in $kJm^{-2}day^{-1}$ with in brackets total irradiated days; Sampled parts of the plant; method of flavonoid analysis;

Species	Percentage increase	Experimental set-up	Reference
Arabidopsis thaliana (Landsberg erecta) wild type (w) and sinapate ester mutant (fah1)	w: 240 %; fah 1: 280 %	C; 2.8 / 0 (24 d); total plant; HPLC	Li *et al.* 1993, Sheahan 1996
Brassica napus	210 %	C; 8.9 / 0 (16 d); leaf; HPLC	Bornman 1997
Brassica napus cv. Paroll (P) and Stallion (S)	cv. P: 250 %; cv S: 170% kaempferol glycosides: cv. P: 135 %; cv. S:110 % quercetin glycosides: P: 4840 %; S: 2530 %	C; 13 / 0 (16 d); 2nd and 3rd leaf; HPLC	Olsson *et al.* 1998
Cryptogramma crispa fern	at: 6; 9; 12; 15; 18; 21 h resp. 130 %; 135 %; 170 %; 185 % (90 mg/g DW); 170 %; 140 %	F; Ambient /reduced UV-B (10 months); single sterile fronds; HPLC	Veit *et al.* 1996
Cucumis sativus cucumber cv. Delikatess	decrease: 65%	C; 7.2/ 0 (10 d); whole seedling; Tevini *et al.* 1981	Tevini *et al.* 1982, Tevini *et al.* 1983a
Cucumis sativus cucumber cv. Delikatess	age: 8; 10; 14; 21 d. resp. 215 % (0.55 mg/g FW); 85 %; 65 %; 40 % (0.3 mg/g FW)	C; 7.9/ 3.4 (8,10,14,21 d); cotyledons of seedlings; Tevini *et al.* 1981	Tevini *et al.* 1983b

Table 1. Continued

Species	Percentage increase	Experimental set-up	Reference
Gnaphalium luteo-album	age: 7; 14; 21; 28 d. resp. gnaphaliin: 100%; 65%; 35%; 35 % calycopterin 155%; 110%; 115%; 115 % 3'methoxy calycopterin 115%; 100%; 160%; 120 %	G; +/-UV-B (7, 14, 21, 28 d); surface compounds on aerial parts of seedlings; HPLC	Cuadra *et al.* 1997
Hordeum vulgare barley	150 %	C; +/-UV-B (7 d); seedlings; Tevini *et al.* 1981	Tevini *et al.* 1981
Hordeum vulgare barley cv. Hege wildtype (w) and pro-antocyanidin mutant (Ant 287)	mut. Ant 287: 7 % of amount of w saponarin w: 125%; mut. Ant 287: 100% lutonarin w: 500% minor flavones compound w:130%	C; 13 / 0 (7 d); primary leaves; HPLC	Reuber *et al.* 1996a
Hordeum vulgare barley cv. Atlas 68	saponarin + lutonarin: 135% (230 nmol/ 4 cm leaf segment)	G; 13.6/ 0 (8 d); primary leaves; HPLC	Liu *et al.* 1995
Marchantia polymorpha	dose: ambient; enhanced resp. luteolin 116 %; 84 % apigenin 120 %; 120 %	G; 25 % enhanced UV-B/ ambient UV-B/ 0 (3 months); -; HPLC	Markham *et al.* 1998a
Petunia Wildtype MP	Age: 29; 39; 45; 53 d. resp. quercetin: 130 %; 267%; 205%; 136 % kaempferol 138%; 163 %; 126 %; 77 %	G; ambient UV-B/ 0 (29, 39, 45, 53 d); whole above ground mass of seedlings; HPLC	Ryan *et al.* 1998
Pinus sylvestris Scots pine	cotyledons; primary leaves resp. 3",6"-di-*p*-coumaroyl-isoquercitrin (DCI) 150% (0.9 mmol/g FW); 300 % (0.2 mmol/g FW) 3",6"-di-*p*-coumaroyl-astragalin (DCA) 120% (1.3 mmol/g FW); 160 % (2.3 mmol/g FW)	C; 4.8/ 0 (42 d); cotyledons and primary leaves; HPLC	Schnitzler *et al.* 1997
Secale cereale rye cv. Custro	isovitexin arabinoside and Isovitexin galactoside: 400 % (24 mg/mg DW epidermis)	C; 32/ 0 (5 d); Epidermis of primary leaves; TLC and HPLC	Tevini *et al.* 1991
Secale cereale rye	epidermis; mesophyll resp. 200 % (257 nmol/ leaf); 85 % (93 nmol/ leaf)	C; 13/ 0 (8 d); previously etiolated seedlings; HPLC	Bornman *et al.* 1997, Reuber *et al.* 1996b

Table 1. Continued

Species	Percentage increase	Experimental set-up	Reference
Phaseolus vulgaris bean	total flavonoids per seedling: decrease: 45%	C; 7.2 / 0 (10 d); seedlings; Tevini *et al.* 1981	Tevini *et al.* 1982, Tevini *et al.* 1983a
Phaseolus vulgaris bean	decrease: 85 %	C; +/-UV-B (8 d); seedlings; (Tevini *et al.* 1981	Tevini *et al.* 1981
Raphanus sativus radish	150%	C; +/-UV-B (5 d); seedlings; Tevini *et al.* 1981	Tevini *et al.* 1981
Raphanus sativus radish	135%	C; 7.2 / 0 (10 d); seedlings; Tevini *et al.* 1981	Tevini *et al.* 1982, Tevini *et al.* 1983a
Raphanus sativus radish	190%	C; 7.2 / 3.4 (8; 10; 14; 21 d); cotyledons; Tevini *et al.* 1981,	Tevini *et al.* 1983b
Silene vulgaris	250%	G; 16.2/ 0 (18 d); seedling; HPLC	Van de Staaij *et al.* 1995

UV-B effects on products of the phenylpropanoid pathway

In response to UV-B, the concentrations of some compounds of the phenylpropanoid pathway are enhanced. Accumulation of pigments may be induced by damaging effects of unnaturally high UV-B doses (general stress response) or by a specific UV-B reaction, which may be triggered by a UV-B photoreceptor (Wellmann 1982).

The examples below show that in most cases the compounds induced by unnaturally high damaging UV-B doses or even UV-C radiation are different from the specific naturally UV-B induced flavonoids and hydroxycinnamic acid (Tables 1, 2 and 3).

Stilbenes and salicylic acid (Figures 1 and 2) are examples of compounds induced in response to different biotic and abiotic stressors among which UV radiation (Dixon & Paiva 1995, Grimmig & Matern 1997, Yalpani *et al.*1994, Zinser *et al.* 1998). Because high doses of UV-B, UV-C or a pathogen are needed for the induction of these specific compounds, they probably do not play an important role in the protection against elevated UV-B in sunlight (Fritzemeier & Kindl 1981, Langcake & Pryce 1977, Schnitzler *et al.* 1997, Zinser *et al.* 1998).

Pterocarpans, furocoumarins and isoflavonoids often show higher production rates in response to phytopathogens than in response to UV-B, therefore the induction of these compounds seems to be a general stress response (Dakora 1995, Dixon & Paiva 1995,

Wellman 1982).

Hydroxycinnamic acids and flavonoids are particularly important phenolics in response to UV-B (Tables 1, 2 and 3). Most research on UV-B absorbing pigments concentrates on the flavonoids only, however, also hydroxycinnamic acids are found in such high concentrations in leaves that they may have a function in UV-B absorption (Table 2).

Table 2. Percentage increase of phenolic acid concentrations in response to UV-B. (control: 100 %). In brackets: absolute concentrations for UV-B treatment.

Species	Percentage increase	Kind of phenolic acids	Reference
Arabidopsis thaliana (Landsberg erecta) wild type (w) and flavonoid mutant (tt4)	w: 130%; tt4: 155 %	sinapate esters	Sheahan 1996; Li *et al.* 1993
Brassica napus	120 % (Highest concentrations and increase in adaxial and abaxial epidermis)	hydroxycinnamic acids	Bornman 1997
Glycine max soybean	125% (131 mg/g FW)	total phenol	Singh 1996
Hordeum vulgare barley	whole leaf; lower epidermis resp. ferulic acid: soluble: 140% (7 nmol/4 cm leaf); 175% (0.7 nmol/4 cm leaf) insoluble: 100% (22 nmol/4 cm leaf); 185% (8.14 nmol/4 cm leaf)	ferulic acid, *p*-coumaric acid, 5-hydroxyferulic acid, sinapic acid	Liu *et al.* 1995
Phaseolus mungo	125% (126 mg/g FW leaf)	total phenol	Singh 1996
Pinus sylvestris Scots pine	wall bound pigments: 4 coumaric acid: 95 % (13 mM) ferulic acid 120% (1.9 mM) lignin 100 % (6.54 mM)		Schnitzler *et al.* 1996
Secale cereale rye	epidermal HCA esters: 100 %: no increase. (Highest concentrations in adaxial and abaxial epidermis)	hydroxycinnamic acid esters	Bornman *et al.* 1997, Reuber *et al.* 1996b
Secale cereale rye	smal increases in epidermis in contrast to flavonoids	cinnamoyl esters	Tevini *et al.* 1991
Triticum aestivum wheat	cinnamic acid, only observed in UV-B treated plants after 4 days treatment		Sharma *et al.* 1997
Vigna radiata	125 % (61.3 mg/g FW leaf)	total phenol	Singh 1996

Table 3. Percentage increase in UV-B absorption of leaf extracts in response to UV-B. (control: 100 %). In brackets absorption calculated on fresh weight (FW), leaf area (LA), total leaf (TL) basis. Analysis: Extraction solution: ethanol (EtOH), methanol (MeOH); Extraction temperature; Extraction time; Wavelength at which absorption was measured.
Growth conditions: Experiment carried out in growth chamber (C), greenhouse (G) or field filter experiment (F); UV-B doses in kJ m^{-2} day^{-1}; in brackets: total number of irradiated days; sampled parts of the plant.

Species	Percentage increase	Analysis	Growth conditions of measured plant material	Reference
Arabidopsis thaliana	At 300; 310; 334 nm resp. 170%; 170%; 160% (FW)	EtOH 80%; 55°C; 30 min; 300, 310, 334 nm	C, C; +/-UV-B (12 d); Total aerial parts	Lois 1994
Brassica rapa	110% (LA)	Acid MeOH 80%; 60°C; 10 min; 300 nm	G; 3.7 /7.4 (38 d); Uppermost fully expanded leaf	Day & Demchik 1996
Bromus hordeaceus	105 % (FW)	Acid MeOH 80%; 90°C; 10 min; 300 nm	G; 9.2 /0 (Just germinated); seedling	Tosserams *et al.* 1997a
Bromus hordeaceus	130 % (FW)	Acid MeOH 80%; 90°C; 10 min; 300 nm	G; 10.6/0 (87 d); Uppermost fully expanded leaf	Tosserams *et al.* 1997b
Bromus sterilis	140% (FW)	Acid MeOH 80%; 90°C; 10 min; 300 nm	G; 10.6/0 (52 d); Uppermost fully expanded leaf	Tosserams *et al.* 1997b
Calamagrostis epigeios	90 % (FW)	Acid MeOH 80%; 90°C; 10 min; 300 nm	G; 10.6 / 0 (66 d); Uppermost fully expanded leaf	Tosserams *et al.* 1997b
Calamagrostis epigeios	21; 36; 57 days UV-B irradiation resp. 100%; 135%; 130% (FW)	Acid EtOH 99%; 90°C; 60 min; 334 nm	F; Ambient /reduced UV-B (21, 36, 57 d); Uppermost fully expanded leaf	Tosserams *et al.* 1996
Cucumis sativus cucumber cv. Marketmore (M) Salad Bush (S) Ashley (A) Poinsett (P)	cv. M 115; cv. S:100 %; cv. A: 110 %; cv. P 110% (DW)	Acid EtOH 99%; 80°C; 10 min; 300 nm	F; Ambient /reduced UV-B (12 d); Third expanded leaf	Krizek *et al.* 1997
Glycine max soybean cv. Clark (C), Harosoy (H) Wildtype (W) and flavonoid mutant (M)	Dose 10.7 / 14.1 kJ/m2/day resp. cv. CW: 110 %; 145 % cv. CM: 90 %; 115 % cv. HW: 140 %; 135 % cv. HM: 175 %; 180 % (LA)	Acid MeOH 80%; 20°C; 72 hrs; 300 nm	G; 10.7/ 14.1/ 0 (10 weeks); Youngest fully expanded leaflet	Middleton & Teramura 1993

Table 3. Continued

Species	Percentage increase	Analysis	Growth conditions of measured plant material	Reference
Gnaphalium luteo-album	After 7; 14; 21; 28 d resp.110%; 150%; 190%; 180% (TL)	Acid MeOH 80%; 20°C; grinding; 300 nm	G; 10.6 / 0 (44 d); Uppermost fully expanded leaf	Cuadra *et al.* 1997
Holcus lanatus	190 % (FW)	Acid MeOH 80%; 90°C; 10 min; 300 nm	G; 9.2 /0 (Just germinated); seedling	Tosserams *et al.* 1997a
Oenothera biennis	90% (FW)	Acid MeOH 80%; 90°C; 60 min; 300 nm	G; 10.6 /0 (45 d); Uppermost fully expanded leaf	Tosserams *et al.* 1997b
Oenothera biennis	95% (FW)	Acid MeOH 80%; 90°C; 10 min; 300 nm	G; 9.2 /0 (Just germinated); seedling	Tosserams *et al.* 1997a
Pisum sativum pea mutant Argenteum	9; 24 days old leaves resp. 185 %; 105% (LA)	Acid MeOH 98%; 80°C; 10 min; 300 nm	F; 4.5/ 2.4 (25 d); 24 (oldest) and 9 (just unfolded) days old leaves	Day *et al.* 1996
Pisum sativum pea cv. JI1389 JI812 JI1176 JI132 scout vedette	leaf buds; fully expanded leaves resp. JI1389: 95%; 100% JI812: 135%; 120% JI1176: 115 %; 110% JI132: 135 %;100% Scout: 115%/ 110% Vedette: 120%/ 110% (FW)	Acid MeOH 80%; 20°C; grinding; 300 nm	G; 6.5/ 0 (32 d); Leaf buds and fully expanded leaves (middle canopy leaves)	González *et al.* 1996
Plantago lanceolata	125 % (FW)	Acid MeOH 80%; 90°C; 60 min; 300 nm	G; 10.6/0; (59 d) Uppermost fully expanded leaf	Tosserams *et al.* 1997b
Plantago lanceolata	After 21; 35 days UV-B irradiation resp.110%,140 % (FW)	Acid EtOH 99%; 90°C; 60 min; 334 nm	F; Ambient /reduced UV-B (21; 35 d); Uppermost fully expanded leaf.	Tosserams *et al.* 1996.
Plantago lanceolata	100 % (FW)	Acid MeOH 80%; 90°C; 10 min; 300 nm	G; 9.2/ 0 (Just germinated plants); seedling	Tosserams *et al.* 1997a

Table 3. Continued

Species	Percentage increase	Analysis	Growth conditions of measured plant material	Reference
Populus trichocarpa and *P. trichocarpa* x *P. deltoides*	LPI 5,10,15 resp. 90%; 105%; 110% (LA)	Acid MeOH 80%; 20°C; grinding; 300 nm	F; Ambient/reduced UV-B (-); leaves with leaf plastochron index 5, 10 and 15	Schumaker *et al.* 1997
Rumex obtusifolius	135% (FW)	Acid MeOH 80%; 90°C; 60 min; 300 nm	G; 10.6 / 0 (44 d); Uppermost fully expanded leaf	Tosserams *et al.* 1997b
Rumex obtusifolius	105 % (FW)	Acid MeOH 80%; 90°C; 10 min; 300 nm	G 9.2 / 0 ; (Just germinated plants); seedling	Tosserams *et al.* 1997a
Senecio jacobaea	105 % (FW)	Acid MeOH 80%; 90°C; 10 min; 300 nm	G 9.2 / 0; (Just germinated plants); seedling	Tosserams *et al.* 1997a
Senecio jacobaea	100 % (FW)	Acid MeOH 80%; 90°C; 60 min.; 300 nm	G; 10.6 / 0 (66 d); Uppermost fully expanded leaf	Tosserams *et al.* 1997b
Silene vulgaris high land population	120 %	MeOH 90% 20°C; 24 hrs 300 nm	G; 16.2/ 0 (18 d); seedlings	Van de Staaij *et al.* 1995
Triticum aestivum wheat cv. HD 2380	3; 5; 7 days old resp. 150%; 160%; 205% (FW)	Acid MeOH; 80%; 20°C; 120 min; 300 nm	C; +/-UV-B; (3, 5, 7 d); seedlings	Sharma *et al.* 1997
Urtica dioica	After 20; 33 days UV-B irradiation resp.110%; 125% (FW)	Acid EtOH 99%; 90°C; 60 min; 334 nm	F; Ambient /reduced UV-B (20, 33 d); Uppermost fully expanded leaf.	Tosserams *et al.* 1996
Verbascum thapsus	130 % (FW)	Acid MeOH 80%; 90°C; 10 min; 300 nm	G 9.2 / 0; (Just germinated plants); seedling	Tosserams *et al.* 1997a
Verbascum thapsus	After 18; 46; 59 days UV-B irradiation resp. 125%; 125%; 150% (FW)	Acid EtOH 99%; 90°C; 60 min; 334 nm	F; Ambient /reduced UV-B (18; 46; 59 d); Uppermost fully expanded leaf.	Tosserams *et al.* 1996
Verbascum thapsus	135% (FW)	Acid MeOH 80%; 90°C; 60 min; 300 nm	G; 10.6 / 0 (50 d); Uppermost fully expanded leaf	Tosserams *et al.* 1997b
Vicia faba faba bean cv. Minica	Dose 4.6; 7.6; 10.6 kJ/m2/day resp. 33 days old plants: 115 %; 130 %; 125 % 60 days old plants:100 %; 85 %; 120 % (FW)	Acid MeOH 80%; 90°C; 60 min; sum of absorption between 280 and 320 nm	G; 4.6/ 7.6/ 10.6/ 0 (33, 60 d); Youngest fully developed leaf	Visser *et al.* 1997

Table 4. Spectral maxima of major classes of phenolics. (Measured in methanolic solvent, except for anthocyanins which are measured in MeOH-HCl) (Harborne 1989)

Class	Spectral maxima (nm)
Simple phenols	266-295
Phenolic acids	235-305
Hydroxycinnamic acids	227-245, 310-332
Hydroxy coumarins	250-260, 280-303, 312-351
Stilbenes	300-310, 320-330
Phenanthrenes	265-270, 272-280, 300-306, 310-315
Flavonoids:	
Isoflavones	255-265, 310-330
Flavanones	275-290, 310-330
Flavones, biflavones	250-270, 330-350
Flavonols	250-270, 350-390
Chalcones	240-260, 365-390
Aurones	240-270, 390-430
Anthocyanins	267-275, 475-545
Xanthones	230-245, 250-265, 305-330, 340-400
Benzoquinones	260-290, 375-410
Naphthoquinones	220-250, 250-290, 330-340, 400-430
Anthraquinones	220-230, 252-260, 267-279, 430-450

UV absorbing properties.

As an adaptation to UV-B, plants can reduce the amounts of UV-B reaching the photosynthetic tissues of the leaves (mesophyll) where UV-B can be damaging. Plants may attenuate the UV-B radiation by morphological and chemical changes. Table 4 shows spectral maxima of the different types of phenolics produced in the phenylpropanoid pathway. As can be seen, the phenolics show high absorbance of UV radiation (Table 4 and 5). In contrast to morphological adaptations, such as altering leaf angle, thicker leaves and increasing reflectance of leaf surfaces by hairs or epicuticular wax crystals, the accumulation of phenolics increases only the UV-B and not the Photosynthetic Active Radiation (PAR) absorption within the leaves (Day *et al.* 1992, DeLucia *et al.* 1992, Krauss *et al.* 1997). Therefore, photosynthesis is not affected (Krauss *et al.* 1997). Comparing the transmittance of radiation with wavelengths between 300 nm and 680 nm through leaves shows that the short wavelengths penetrate less deep in leaves or needles than the longer wavelengths, indicating that the penetration distance of UV radiation in leaves is short compared to PAR (Day *et al.* 1992, DeLucia *et al.* 1992). To

assess the UV-B protection by phenolics in the leaves, it is necessary to explore the absorption spectrum of the protective compounds.

However, the spectral maxima shown in Table 4 do not give absolute information about the absorption capacity, because we do not know the extinction coefficients. Unfortunately, only the wavelength range with the best absorption capacity for one group of compounds is known. With this information, it is not possible to compare the different groups for absolute absorption capacity. Another problem in interpreting these data is the nature of the solvent, which is used to measure the absorption maxima. Phenolics that act as a UV-B screen are often found in the vacuole. We did not find many data on spectra of these compounds in water or cell sap solutions with a pH equal to that of the vacuole. Figure 4 gives absorption spectra of flavonoids and sinapate esters, solved in a physiologically relevant solvent. It seems useful to publish more of these kinds of measurements on flavonoids and hydroxycinnamic acids.

Besides the absorption spectrum of phenolics, also the localisation of phenolics within the leaves is important in the actual UV-B screening effectiveness of these compounds. If phenolics are accumulated in the epidermis or are enhanced only in the epidermis after UV-B treatment they function as a shield for the photosynthetic tissue located deeper within the leaves. For this reason it seems to be useful, to calculate the amount of biologically effective UV-B radiation reaching the mesophyll to assess the potentially damaging UV-B effect within the leaves.

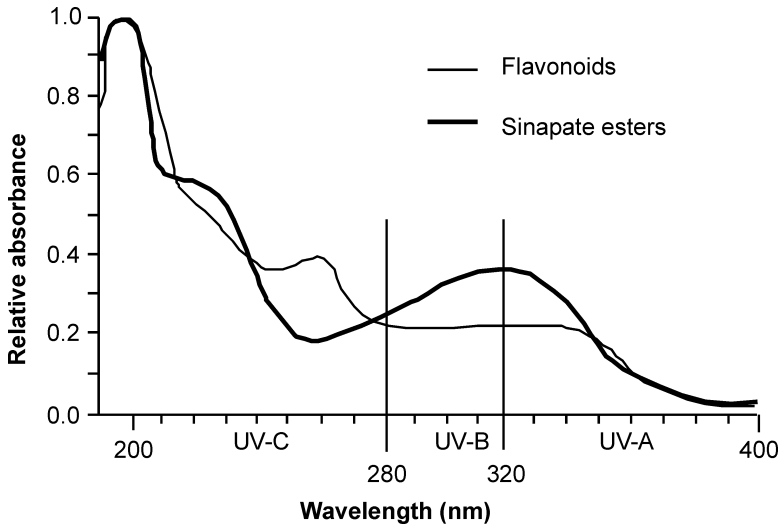

Figure 4. Absorption spectra of flavonoids and sinapate esters in aqueous solution at vacuolar pH. The maximum at 200 nm is normalised to 1. (Modified after Sheahan 1996)

Increasing concentrations of phenolics also enhances UV-B absorption. According to Beer's law, there is a linear relationship between the concentration of compounds and light absorption in a solution. Thus the UV-B sensitivity of leaves and whole plants is strongly correlated with the concentrations of constitutive and UV-B induced flavonoids (González *et al.* 1996, Lois 1994).

The structure of the phenolics in the leaves determines the absorption spectrum. Changes in structure may change the absorption spectrum. When the absorption maxima shift towards the UV-B part of the spectrum, the effectiveness for UV-B absorption increases. We give some correlations between the structure and the absorption of a compound. The simple phenolics have one absorption maximum, while the flavonoids have two or more absorption maxima. Maximum I is in the range 300-400 nm and the value is determined by the A-ring benzoyl system (Figure 3). Maximum II is determined by the B-ring cinnamoyl system and is in the range 240-285 nm, thus nearly not relevant for UV-B radiation in the field. Substitution of the different C atoms can shift the absorption maxima. Oxygen substitutes in the B ring of flavonols shift the absorption to the longer wavelength of band I, whereas oxygenation in the A-ring shifts band II. Methylation or glycosylation of the 3-, 5-, and 4'-hydroxyl groups leads often to shifts in band I (Markham 1982). Table 5 gives spectral maxima of various kaempferol and quercetin compounds showing the effect of a hydroxyl group in the B ring and the shift in absorption maxima in case of glycosylation (Markham 1982). Apart from the substitution and oxygenation of the flavonoid skeleton, the presence of metals, the pH and the

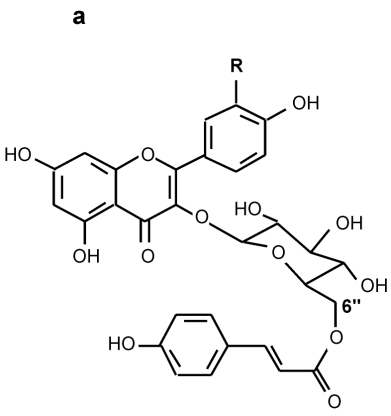

Figure 5a. Monoacylated flavonols occurring in Scots pine. R=OH: 6"-O-(*para*-coumaroyl) isoquercitrin. R=H: 6"-O-(*para*-coumaroyl) astragalin. (Modified after Jungblut *et al.* 1995).

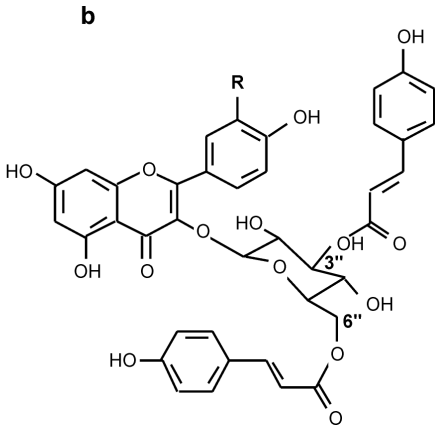

Figure 5b. Diacylated flavonols occurring in Scots pine. R=OH: 3",6"-di-O-(*para*-coumaroyl) isoquercitrin. R=H: 3",6"-di-O-(*para*-coumaroyl) astragalin. (Modified after Jungblut *et al.* 1995).

Table 5. Spectral maxima of kaempferol and quercetin aglycones and glycosides. (Measured in methanolic solvent) (based on Markham 1982). Sh means shoulder.

Flavonoid	Spectral maxima (nm)		
	UV-C	UV-B	UV-A
Kaempferol (3,5,7,3,4' -OH)	266	294 (sh)	322 (sh), 367
Kaempferol 3-O-glucoside	264	301 (sh)	350
Kaempferol 7-O-glucoside	264		321, 362
Kaempferol 3,7-O-glucoside	265		323 (sh), 350
Quercetin (3,5,7,3',4' -OH)	255, 269 (sh)	301 (sh)	370
Quercetin 3-O-glucoside	257, 269 (sh)	299 (sh)	362
Quercetin 7-O-rhamnoside	256, 269 (sh)		372
Quercetin 3,7-O-glucoside	256, 268 (sh)		355

interaction between different flavonoids can shift the absorption maxima (Markham 1982, Shirley 1996).

In response to UV-B radiation the increase in concentration of flavonoids seems to be higher than the increase in hydroxycinnamic acids (Table 1 and 2). However, the hydroxycinnamic acids may be at least as important as the flavonoids in scavenging UV-B radiation, because of the higher extinction coefficient in the UV-B range. Sinapate esters (hydroxycinnamic derivates) have a stronger absorption in the UV-B range compared to the flavonoids (Figure 4). Spectral analyses of the diacylated flavonoid molecules in Scots pine (Figure 5b) suggests that the hydroxycinnamic part of this molecule represents the crucial UV-B chromophore because of the stronger absorbance of coumaryl in the UV-B part of the spectrum (Landry *et al.* 1995). Jungblut and co-workers (1995) showed with UV spectra normalised at 350 nm, that the UV-B absorption capacity of the monoacylated flavonol glycosides is twice as high as the non-acylated flavonols. The diacylated flavonols absorb a factor three better than the non-acylated flavonols (Figs. 5a and 5b for an example of a mono- and diacylated flavonol).

Models how a UV-B screen is provided by flavonoids and hydroxycinnamic acids.

According to the literature flavonoids and hydroxycinnamic acids can act as a UV-B screen in leaves through different mechanisms. For some species both branches of the phenylpropanoid pathway integrate and overlap to provide a multiple defence mechanism against UV-B radiation. The flavonoids only partly contribute to the absorption of UV-B. Also other UV-B absorbing compounds such as phenolic acids play a role in the UV-B absorbance of leaves exposed to UV-B radiation (Landry *et al.* 1995, Li *et al.* 1993, Lois 1994, Reuber *et al.* 1996a, Sheahan 1996, Shirley 1996, van de Staaij *et al.* 1995). Table 2 and 3 show that mutants of *Glycine max* and *Arabidopsis thaliana,* which are not capable of flavonoid synthesis have enhanced UV-B absorption in response to UV-B. Under "normal" circumstances, both groups of compounds contribute to UV-B absorption. When one group of compounds is not synthesized, for example because of a mutation, there are still compounds from the other group available to offer protection against UV-B radiation (Middleton & Teramura 1993, Shirley 1996). These alternative UV-B absorbing compounds in *Arabidopsis* may be sinapate esters (Li *et al.* 1993).

Another possible UV-B screening function of phenolics is that both flavonoids and hydroxycinnamic acids are combined into one molecule to provide a UV-B shield. This occurs in *Pinus sylvestris* and *Picea abies* where mono- and diacylated flavonols serve as UV-B absorbing pigments in the needles (Figure 5) (Hutzler *et al.* 1998, Schnitzler *et al.* 1997). In angiosperms, examples of compounds, which are composed of hydroxycinnamic acids and flavonoids are also known. *Brassica napus*, *Pisum sativum* and *Oryza sativa* contain compounds with kaempferol and quercetin glycosides and *p*-coumaryl, ferulyl and/or sinapyl derivates (Harborne 1980, Markham *et al.* 1998b, Olsson *et al.* 1998, Wilson *et al.* 1998). However, Wilson and co-workers (1998) found only moderate amounts of acylated flavonoids present in UV-B exposed leaves.

Further, it is possible that the flavonoids and the hydroxycinnamic acids play a different role in the screening of UV-B. Reuber and co-workers (1996b) suggested that the hydroxycinnamic acids form a constitutive shield, whereas the flavonoid shield is UV-B inducible. Table 1 and 2 show that in response to UV-B, the concentration of flavonoids is enhanced to a greater extent than those of the hydroxycinnamic acids (esters).

Finally, it is possible that, depending on the tissue layer, either the hydroxycinnamic acids or the flavonoids serve as UV-B absorbing compound. Cuadra and co-workers (1997) showed that in response to UV-B, *Gnaphalium luteo-album* have increased levels of highly methylated flavonols on the leaf surface, whereas the internal UV-B absorbing compounds (caffeic acid esters), also increased in response to UV-B.

Methodology for assessing kind and quantity of UV-B absorbing compounds in leaves

The increase in concentration of UV-B absorbing compounds in leaves is a very common response to UV-B (Table 3). To assess the UV-B absorbing capacity of leaves, many *in vivo* and *in vitro* methods are used. The *in vivo* method in which the UV-B radiation in leaves and needles is measured, using fiber optics, is appropriate to assess the total absorbing capacity and for investigating the contribution of different layers to UV-B absorption (Bornman, 1999, Alenius *et al.* 1995, Day 1993, Day *et al.* 1992, DeLucia *et al.* 1992). With *in vivo* methods, UV-B absorption including chemical parameters as well as the physical parameters (leaf thickness and reflectance) is measured. These *in vivo* measurements give an overall indication of UV-B absorbing capacity of the leaf or epidermis but do not give an answer to the question how important the chemical and physical characteristics separately are for the UV-B absorbing capacity.

Using microscopic techniques such as confocal laser scanning microscopy, fluorescence microscopy and electron microscopy, the localisation of flavonoids within tissues and even within cells can be studied. The hydroxycinnamic acids have autofluorescent properties and flavonoids fluorescence after dying with ammonia or Naturstoffreagenz A (Gitz *et al.* 1998, Hutzler *et al.* 1998, Karabourniotis *et al.* 1998, Schnitzler *et al.* 1996). With *in situ* hybridisation and immunolocalisation techniques, the localisation of the specific enzymes of the phenylpropanoid pathway can be studied (Beerhues *et al.* 1988, Dixon & Paiva 1995, Grandmaison & Ibrahim 1996, Zinser *et al.* 1998).

To estimate quantity and kind of UV-B absorbing compounds and their importance in the UV-B absorbing capacity, analyses of leaf tissue extracts are necessary. A method often used to measure relative amounts of total UV-B absorbing compounds in leaves is extraction in hot acid methanol (e.g. methanol: water: HCl (79:20:1). The absorption of the extract is measured spectrophotometrically at 300 nm. The absorbance is calculated based on leaf mass or leaf area and is given as a relative value. Some examples of UV-B effects on the absorption of leaf extracts and the various methods used to extract the absorbing compounds are given in Table 3.

A variation on this method is described by Tevini and co-workers (1981). They also extracted in methanol and measured spectrophotometrically, but they measure the $AlCl_3$ shifted flavonoids only. Adding some drops of $AlCl_3$ solution will shift the absorption maxima of a lot of flavonoids (and in most cases not the other phenolics) (Markham &

Mabry 1975). With this method, it is possible to distinguish the increase in flavonoid concentration from increases in absorption caused by the other phenolics.

To assess the amount of flavonoids and other phenolics separately, also chromatographic methods are used. General procedures to elucidate the type of phenolics in a sample are described by Harborne (1989). The type of flavonoids in plants can be elucidated using various thin layer and paper chromatography systems. Markham (1982) describes a structural and detailed method to determine the nature of the flavonoids in the sample. Other advanced analytical methods such as NMR, mass spectroscopy and UV spectroscopy are used for structure elucidation as well.

Quantitative HPLC methods of flavonoid analysis are available abundantly. For this type of analysis, the water-soluble flavonoids are often extracted in methanol (various concentrations). In some cases, purification steps are taken to remove chlorophyll and other secondary compounds. To separate flavonoid compounds in an extract, a C18 column is applied with an acid eluens gradient (for example acidified water and acetonitril). In general, a photodiode array detector is applied, to determine absorption spectra of the different peaks (cf. references in Table 1). To quantify flavonoids in specific parts of the leaf, samples are taken using biochemical techniques. It is possible to make protoplasts of guard cells, mesophyll cells and epidermal cells. Another possibility is to make paradermal sections of the leaf to analyse flavonoids in the different layers. The flavonoids in these protoplast or coupes can be analysed by HPLC (Bornman *et al.* 1997, Schulz & Weissenböck 1986, Weissenböck *et al.* 1984, Weissenböck *et al.* 1986).

Tables 1, 2 and 3 show the enhancement of respectively flavonoids (assessed by HPLC and spectrophotometer using the $AlCl_3$ reagents), of the phenolic acids (assessed by HPLC), and the total amount of UV-B absorbing compounds (crude extract, measured at 300 nm) caused by UV-B. It is difficult to compare these results because a crude acidified methanol extract contains all methanolic soluble compounds including the compounds, which are not involved in a UV-B response.

For example the results of van de Staaij and co-workers (1995) in Tables 1 and 3 show approximately 250 % increase in the concentration of the total flavonoids measured with HPLC, but only 120 % increase in absorption of the total UV-B absorption in a crude extract (with flavonoids and other UV-B absorbing compounds). Comparing these two methods is difficult because the 'background absorption' (absorption of the extract apart from phenolics) cannot be estimated in the crude extract. With the crude extraction method, the enhancement of phenolics is underestimated. Comparison of Table 1 and 3 also shows lower values for the crude extraction method.

Factors influencing the kind and quantity of phenolics

The accumulation of flavonoids in response to UV-B is very common for many plant species (Shirley 1996, Tevini & Teramura 1989, Table 3). It is known that many phenolic esters such as sinapate esters also accumulate in response to UV irradiation (Alenius *et al.* 1995, Chapple *et al.* 1992, Fischbach *et al.* 1999, Landry *et al.* 1995, Li *et al.* 1993, Sheahan 1996, Table 2). However, the variation in quantity and quality of the flavonoids between different species of higher plants is very high. We will discuss some sources, which may cause this variation.

Life form, species and cultivar dependency.

In many herbaceous species UV-B radiation is only partly absorbed in the upper epidermis, so that a part of this radiation penetrates into the mesophyll in contrast to perennial and woody plants (Day *et al.* 1992, Day 1993, Krauss *et al.* 1997). Conifers are more efficient in screening out UV-B than herbaceous plants when comparing the absorbing capacity across the leaf or needle in transversal direction (Day *et al.* 1992, Day 1993). Thus, conifers can produce more effective UV-B absorbing pigments in the epidermis than herbaceous plants and/or the localisation of these pigments is more efficient. A reason for this variation in absorbing capacity may be the difference in lifespan. The prevention of UV-B damage in the mesophyll is essential for long-lived leaves, since no seasonal renewal of these tissues occurs (Krauss *et al.* 1997, Stephanou & Manetas 1997).

In various plant species, different kinds of flavonoids increase in concentration in response to UV-B (Table 1). Related plant species tend to synthesize the same type of flavonoids. However, the amount of both constitutive and UV-B inducible flavonoids can vary from species to species (Bornman *et al.* 1997, Table 1). Also differences in flavonoid concentration within one species are found. Rice cultivars, sensitive to UV-B showed lower levels of flavonoids than tolerant cultivars. Tolerant *Oryza sativa* cultivars contain higher levels of phenolics when exposed to UV-B (Caasi-Lit *et al.* 1997). However, Krizek & co- workers (1997) did not find differences in flavonoid content between differentially sensitive cultivars of *Cucumis sativus*.

Localisation within the leaf.

a. Organ level.

Within one plant, differences in kind and quantity of flavonoids are found between several organs like the cork layer, leaves and roots (Hayashi *et al.* 1996, Wollenweber & Dietz 1981). It is not surprising that flavonoids differ between organs because flavonoids have various functions in several organs (see elsewhere in this chapter).

Differences in flavonoid composition (kaempferol and quercetin derivates) are found between cotyledons and primary leaves of *Pinus sylvestris* after exposure to UV-B (Table 1, Schnitzler *et al.* 1997). Different concentrations and kinds of flavonoids are found in various leaves of *Arabidopsis thaliana* and *V. faba* as well (Charest *et al.* 1986, Lois 1994, Chapter 4 and 5 of this thesis). However, comparable flavonoid type and concentration are found in apical and lower leaves of *Chrysosplenium americanum* (Charest *et al.* 1986).

A reason for this variation between leaves can be the difference in developmental stage. The constitutive difference in the amount of flavonoids in the leaves is also dependent on the overall developmental program of the plant (Lois 1994). It is also possible that cotyledons have flavonoids carried over from seeds to provide a UV screen for the seed and the young seedlings (Gitz *et al.* 1998).

b. Tissue level.

Besides variation in kind and concentration of flavonoids between leaves, there are also differences within the leaf. The cuticle is the first barrier where UV-B light is attenuated by pigments associated with the cutin matrix and wax layer. The cuticular attenuation of UV-B radiation of woody and perennial species exceeds that of annual herbaceous species, whereas in the visible range (PAR, 400-700 nm) cuticular transmittance remains very high (Day *et al.* 1992, Krauss *et al.* 1997). Flavonols as well as hydroxycinnamic acids may be responsible for the surface attenuation of UV-B irradiation (Cuadra *et al.* 1997, Krauss *et al.* 1997). Perhaps the UV-B attenuation is constitutive in the cuticle and not UV-B inducible (Hutzler *et al.* 1998). However, the function of flavonoids in the cuticle as UV-B screen does not seem to be a general UV-B adaptation, although UV-B absorbing pigments are found in this layer too. No correlation between UV-B sensitivity and UV-B absorbing compounds in the epicuticular wax layer is found in *Dittrichia viscosa* (Stephanou & Manetas 1995).

It seems that the phenolics in the epidermal layer are more crucial as a UV-B adaptation than the cuticle. The epidermal transmittance of UV-B radiation can vary between 0- 40 % of the incident UV-B light on the leaf surface (Day *et al.* 1993). Alenius

and co-workers (1995) showed that the UV-B absorbing pigments of *Brassica napus* were concentrated in the upper 40 μm of the leaves. Also in other cultivated herbaceous plants, the flavonols and anthocyanins or enzymes necessary for flavonoid production, are mainly found in the epidermal layer (Beerhues 1988, Hrazdina *et al.* 1982, Weissenböck *et al.* 1984, Weissenböck *et al.* 1986). Woody and perennial species show a far better attenuation of UV-B in the upper layer of the leaves than herbaceous plants (Day *et al.* 1992).

The UV-B induced flavonoids are often accumulated near the induction site, i.e. in the epidermal cells. It seems that hardly any signal transduction to other tissues (cells) occurs (Dixon *et al.* 1995, Lois 1994, Hrazdina *et al.* 1982). Probably the transport of flavonoids in plants is restricted to cells and there is no transport within the vascular system of plants or between tissues. Therefore, the tissues in which flavonoid accumulation is found are also the sites of synthesis of these flavonoids (Hrazdina *et al.* 1982, McClure 1975).

Even restriction of flavonoids to certain cells within a tissue is a common feature (McClure 1975). Weissenböck and co-workers (1984) showed in faba bean similar or higher concentrations of flavonoids in guard cells with the UV-B sensitive chloroplasts compared to the epidermal cells, which have no chloroplasts. The non-uniform distribution of the flavonoids in the epidermis of some herbaceous species gives locally high UV-B transmittance. Transmittance through the stomatal pores is relatively high, whereas transmittance through stomatal guard cells is low (Day *et al.* 1993).

Besides flavonoids in the epidermis also flavonoids, or enzymes of the phenylpropanoid pathway are also found to a lesser extent in the mesophyll, whereas the "factory" of UV-B induced flavonoids seems to be in the epidermal layer (Beerhues *et al.* 1988, Hrazdina *et al.* 1982, Schulz & Weissenböck 1986). There are often differences in quality and quantity in flavonoids of mesophyll and epidermis cells (Bornman *et al.* 1997, Schulz & Weissenböck 1986, Weissenböck *et al.* 1984). Some mesophyll flavonoids, such as the flavanes and non-acylated flavonol glycosides in Scots pine are constitutive and not UV-B inducible (Schnitzler *et al.* 1996). Apart from the UV-B screening function, flavonoids can have many other functions in plant tissues. The flavonoid pathway is also involved in the production of compounds such as lignin and proanthocyanins, which are the precursors of tannins (Hahlbrock 1981, Hrazdina *et al.* 1982, McClure 1975, Shirley 1996). Schnitzler and co-workers (1996) concluded that the phenolic compounds in the mesophyll are not important in UV-B protection. Maybe this conclusion can be extended more generally to woody and perennial plants.

c. Subcellular level

It is supposed that flavonoids are synthesized in metabolic clusters, which are called metabolons, located in the cytoplasm and in loose association with membranes of the plastids (Beerhues *et al.* 1988, Dixon *et al.* 1995, Harborne 1980, Hrazdina *et al.* 1982, McClure 1975). Konishi and co-workers (1996) found that only the cytosolic form of ACCase (Acetyl-CoA Carboxylase, to synthesize malonyl CoA) is induced by UV-B, whereas the plastidic ACCase is not induced by UV-B radiation. It is possible that the plastidic ACCase is needed for the constitutive, non-UV-B inducible flavonoids or for non-flavonoid synthesis. Plastidic ACCase may be used for repair of UV-B damage to fatty acids (Konishi *et al.* 1996).

Different subcellular distribution patterns of flavonoid metabolites are observed for leaves of annual and evergreen plants (Hutzler *et al.* 1998). The final conjugation of the sugars to the flavonoids seems to take place when the aglycone is transported over the tonoplast into the vacuole (Dixon *et al.* 1995, Harborne 1980). Transmittance measurements, confocal laser scanning and fluorescence microscopy analyses indicate that the hydrophilic flavonoid glycosides accumulate mainly in the vacuole (Day *et al.* 1993, Hrazdina *et al.* 1982, Hutzler *et al.* 1998, Schnitzler *et al.* 1996). Flavonoids such as flavonol sulphate esters were found in the cytosol of *Flaveria chloraefolia* (Grandmaison & Ibrahim 1996).

Flavonoids in maize offer UV-B protection by preventing DNA damage (Stapleton & Walbot 1994). For this reason, in some plant species especially flavonoids, found in the nuclear region, may protect DNA (Day *et al.* 1993, Grandmaison & Ibrahim 1996, Hutzler *et al.* 1998, Karabourniotis *et al.* 1998, Sheahan 1996). It seems that DNA protection by flavonoids is depending on the plant species. However, it is still not clear whether the DNA is directly protected from UV-B injury by nuclear flavonoids.

The localisation of phenolics can also change during development. Young developing multicellular leaf hairs of *Dittrichia viscosa* show high concentrations of polyphenolics in the cytoplasm. When the hairs mature, synchronously with secondary wall thickening, deposition of polyphenolics on the cell wall and cuticle occurs and phenolics in the cytoplasm disappear (Karabourniotis *et al.* 1998).

Additionally, many UV-B absorbing phenolics are found outside the cell in the cuticle, cell wall and periplasmic area of different plant species of all life forms (Charest *et al.* 1986, Day *et al.* 1993, Hutzler *et al.* 1998, Karabourniotis *et al.* 1998, Schnitzler *et al.* 1996). For pine needles such as those of *Picea abies* and *Pinus sylvestris*, it seems that *p*-coumaric acid and kaempferol-3-glycoside are the most effective cell wall bound screening pigments, whereas ferulic acid and lignin seem to play a minor role in UV-B screening (Fischbach *et al.* 1999, Schnitzler *et al.* 1996). In rye, Hutzler and co-workers

(1998) found ferulic acid to be bound to the outer periclinal and anticlinal epidermal cell walls.

It seems that these UV-B absorbing compounds in the cuticle and periclinal cell wall are mainly constitutive in most plant species. However, differences between annual and perennial herbaceous and woody species and between *plus* and *minus* UV-B treated plants are found (Day *et al.* 1993, Fischbach *et al.* 1999). In herbaceous species water-soluble phenolics in the vacuole are more important than the cell wall bound phenolics in the attenuation of UV-B radiation (Day *et al.* 1993, Schnitzler *et al.* 1996). In *Smilacina stellata* the transmittance under the epidermal protoplasts (including the vacuole with accumulated flavonoids) is less than 10 %, whereas the transmittance beneath the anticlinal cell walls is about 90 % (Day *et al.* 1993).

For woody plants such as *Picea pungens*, Day and co-workers (1993) found a more uniform filter against UV-B radiation. The UV-B absorbing compounds in the cell wall of the leaf are present in such high concentration that there is always a uniform UV-B screen. Overall, we can conclude that in most herbaceous species the screening components are mainly located in the protoplasts of epidermal and guard cells. In most woody and perennial plants, in addition to flavonoids in the protoplast, significant constitutive or UV enhanced amounts are observed extra cellular, bound to cell walls (Day *et al.* 1993, Fischbach *et al.* 1999, Hutzler *et al.* 1998). This combination provides a much more laterally uniform screen.

Time and developmental stage.

Besides spatial factors, there are developmental and time dependent factors, which influence the quantity and quality of phenolics in plants. During growth, the plant and the maturing leaves go through a developmental program with on every moment characteristic kinds and concentrations of compounds. Environmental factors such as UV-B interact with the phenylpropanoid pathway, which results in a particular distribution pattern of phenolics in the plant (Dixon & Paiva 1995). When leaves mature and age, changes in the quantity and quality of phenolics take place, resulting in an altered response to UV-B radiation. The amount of constitutive and UV-B inducible flavonoids and their distribution through the leaf and plant is dependent on the developmental stage.

Early in the development of tissues, most of the cell-specific (constitutive) and UV-B induced flavonoid accumulation occurs because young tissues are metabolically most active (Beerhues *et al.* 1988, Lois 1994, McClure 1975). Thus, the concentration of UV-B absorbing compounds seems to be building up in a maturing leaf (Day *et al.* 1996,

DeLucia *et al.* 1992). Later, the phenolic levels remain at a constant level or decrease (Liu *et al.* 1995, Schulz & Weissenböck 1986). It is suggested that the flavonoids photodegrade after a period of UV-B exposure, so that UV absorption may decrease (Liu *et al.* 1995). The turnover of UV-B absorbing compounds in the cuticle is presumably very low (Krauss *et al.* 1997).

In addition, the distribution of phenolics in the leaves changes with the aging of the leaves. In still unfolded pea leaves, the flavonoid concentration in the lower epidermis (exposed to UV-B radiation) is higher than when the leaves are mature and the lower epidermis is no longer exposed to direct UV-B light. The flavonoid concentration of the upper epidermis and mesophyll increases with the maturation of the leaves (Day *et al.* 1996). In some woody species a shift in localisation of polyphenolics in leaf hairs occurs. Initially, they are located in the cytoplasm. Later, during secondary cell wall thickening, the phenolics are observed in the cell walls (Karabourniotis *et al.* 1998). With the leaf maturation, the contribution of the flavonoids to the total UV-B absorption decreases (Lois 1994, van de Staaij *et al.* 1995).

In a natural environment, where plant development occurs synchronously with the season, changing patterns of phenolics in (parts of) the plant can be caused by the developmental program of the plant, and may be simultaneously modulated by seasonal patterns of abiotic factors such as UV-B. Fischbach and co-workers (1999) calculated that at the beginning of the growing season in June a steep increase in epidermal absorbance of Norway spruce needles takes place, which remains at this level during the rest of the season. Also in *Dittrichia viscosa* seasonal fluctuations in UV-B absorbing compounds of the epicuticle are found with a maximal absorbance in summer (Stephanou & Manetas 1997). In mountain birch the content of total phenolics declines during the growing season. This may be caused by a decreasing rate of biosynthesis, the conversion into insoluble cell wall-bound components and an active conversion into polymeric compounds (lignin and tannin, cf. Figure 2). A seasonal variation is especially found in the non-flavonoid phenolics. The variation in flavonoids is small, compared to the between-tree variation (Nurmi *et al.* 1996). Fischbach and co-workers (1999) showed that the seasonal accumulation of the hydroxycinnamic acids plays a minor role in UV-B protection because the accumulation of these compounds starts in late summer.

It seems that especially the leaf age effects on the soluble UV-B absorbing compounds are much higher than the UV-B effects (Day *et al.* 1996). However, diurnal patterns in soluble flavonoids, triggered by UV-B, are found in field studies with *Anacardium excelsum* and *Cryptogramma crispa*. It is still not clear whether there is a degradation of flavonoids or an inter-conversion of soluble and insoluble flavonoids. In the latter case, the UV-B absorbing capacity of the leaves remains high (Veit *et al.* 1996).

Fischbach and co-workers (1999) showed a seasonal accumulation of UV-B screening pigments in needles of *Picea abies*. The UV-B effect on the accumulation of soluble diacylated flavonols was only observed during one of two seasons, whereas there was an increase in cell wall-bound *p*-coumaric acid and kaempferol-3-O glycoside for both seasons in response to UV-B.

Thus, the first step in developing tissues may be to synthesize flavonoids. The second step is the translocation of flavonoids from cytoplasm to cell wall. Soluble flavonoids seem to be converted into insoluble compounds.

Spectral distribution and light intensity.

In response to UV-B, plants may increase the synthesis of only a selection of the flavonoids present under ambient UV-B radiation. Van de Staaij and co-workers (1995) found that the concentration of some flavonoids increased with a factor of nearly 10, whereas the concentration of other flavonoids did not show enhanced synthesis. Different increases in concentration for different types of flavonoids under elevated UV-B fluxes are also reported by Cuadra *et al.* (1997), Schnitzler *et al.* (1997) and Wilson *et al.* (1998). The specific increase of flavonoids with an additional *ortho*-dihydroxyl group in the B ring in response to UV-B is found in different plant species. Spectrally, these compounds do not differ very much in the UV-B range (Bornman *et al.* 1997, Liu *et al.* 1995, Markham *et al.* 1998a, Markham *et al.* 1998b, Middleton & Teramura 1993, Olsson *et al.* 1998, Reuber *et al.* 1996a, Ryan *et al.* 1998, Schnitzler *et al.* 1997). In the next section we discuss other advantages of the presence of compounds for UV-B protection.

In *Brassica napus* only quercetin and kaempferol di- and triglycosides are strongly accumulated in response to UV-B. However, also different glycosides of quercetin and kaempferol are equally effective in screening UV-B radiation. Perhaps the plant is not selective in accumulation of specific flavonoids in response to UV-B, but the plant tries to minimize the part of the phenylpropanoid pathway that must be upregulated (Wilson *et al.* 1998).

In *Lactuca sativa*, *Hordeum vulgare* and *V. faba*, the effect of UV-A radiation on the flavonoid accumulation seems to be more restricted compared to the effect of UV-B radiation (Krizek *et al.* 1998, Liu *et al.* 1995, Chapter 4 of this thesis). Wilson and co-workers (1998) found that in *Brassica napus*, both UV-B and UV-A radiation are required for induction of specific flavonoids but UV-A radiation alone is sufficient to cause a decrease in some other flavonoids. The mechanism involved is still unclear.

Furthermore, the accumulation of flavonoids is dependent on UV intensity. In *Arabidopsis thaliana,* the accumulation starts 7 hours after the onset of the UV-B

radiation but the increment is steeper when the fluency rate is higher (Lois 1994). Also Tosserams and co-workers (1997a) found a dose dependent increase in UV-B absorbing compounds for seedlings of some dune grassland species. The rule that with increasing UV-B dose, the flavonoid levels become higher, may be valid until a certain UV-B level is achieved. Above this UV-B dose, the flavonoid content decreases probably caused by damage of general cell metabolism (Lois 1994, Tosserams & Rozema 1995, Visser *et al.* 1997). Tosserams and Rozema (1995) found that in *Calamagrostis epigeios,* flavonoids decreased above a dose of 10 UV-B_{BE} kJ m^{-2} day^{-1}. González and co-workers (1998) investigated UV-B absorbing compounds in pea lines at different UV-B doses (2-10 kJ m^{-2} day^{-1}) but did not find a threshold or decrease in UV-B absorbing pigment concentration at higher UV-B doses. However, Markham and co-workers (1998a) did not find an increase of flavones concentration in the enhanced UV-B treatment of *Marchantia polymorpha* compared to the ambient UV-B treatment.

The physiological and ecological implications of increasing hydroxycinnamic acids and flavonoids.

UV-B radiation also causes indirect effects. Changes in UV-B absorption in the leaves can take place due to qualitative and quantitative changes in phenolics and by an altered localisation of the phenolics. So far, the consequences of UV-B induced increases of concentrations of hydroxycinnamic acids and flavonoids have been discussed with regard to the UV-B absorbing properties. However, some of these compounds do not only play a role in the UV screening of the leaves, but also have other important functions for plants, which makes the impact of increasing concentrations of these compounds much higher. Besides this, altering concentrations of phenolics, not caused by UV-B but by other environmental factors, can have a positive effect on the UV-B absorbing capacity of the leaf. We will discuss some ecological and physiological functions of flavonoids and hydroxycinnamic acids and discuss some aspects like the antioxidant function of flavonoids and growth regulation altered by phenolics, in detail.

<u>Functions of flavonoids.</u>

Many flavonoids are induced in response to environmental stresses other than UV-B irradiation such as herbivory, pathogenesis, low nutrient availability, wounding and low temperature (Dixon & Paiva 1995). As a reaction to different stresses, plants synthesize different flavonoids, so there may be competition between pathways leading to flavonoids for UV-B protection (kaempferol, quercetin) or to flavonoids used as anti-fungal agent

(e.g. resveratrol, pterocarpan). It seems that the metabolically highly active tissues, such as primary needles have to cope with competition between different pathways (Schnitzler *et al.* 1997).

Functions of flavonoids in plants include:

1. Signalling molecule for plant-microbe interaction such as nodule formation in the *Rhizobium-Leguminosae* interaction (Dakora 1995, Dixon *et al.* 1996, Shirley 1996). Particular flavonoids excreted by seed coats during imbibition and by roots of seedlings, induce the transcription of nodulation genes in *Rhizobium* cells, necessary to form root nodules for nitrogen fixation (Dakora 1995).
2. Fertility of pollen (Shirley 1996). Some flavonols can serve as signal for pollen and stimulate germination (Dakora 1995).
3. Repellent for insects. Some isoflavonoids, which are abundant in leaves, serve as antifeedant for phytophagous insects (Dakora 1995).
4. Phytoalexin for phytopathogens. In reaction to infection by microbiological pathogens, host plant tissues accumulate isoflavonoids, which function as phytoalexin (Dakora 1995).
5. Colour or odour of flowers to attract insects which provide pollination. Because flavonoids absorb in the UV region and are located in the inner petal and upper part of the corolla, they may act as honey or nectar signal (Dakora 1995, Shirley 1996, Yamasaki 1997).
6. Antioxidant compound to scavenge ROS. UV-B can cause higher levels of ROS, which can generate oxidative stress and is thus harmful in cells (Greenberg *et al.* 1997, Jansen *et al.* 1998). Not each flavonol has the same antioxidant activity. Parts of the flavonoid skeleton, which have high antioxidant activity are the *ortho* 3'4'-dihydroxy part in the B-ring, the *meta* 5,7-dihydroxy groups in the A-ring and the 2,3-double bond in combination with the 4-*keto* group and the 3-hydroxyl group in the C-ring when the B ring has two hydroxy groups (Figure 3). Thus, the flavonoids with *ortho*-dihydroxyl group such as quercetin and luteolin are more effective in dissipating UV-B energy. Many researchers find much higher UV-B induced increases of flavonoid derivates with an additional *ortho*-dihydroxyl group in comparison with flavonoids without this group, which suggests that the anti-oxidant function of the flavonoids is crucial in UV-B protection (Liu *et al.* 1995, Markham *et al.* 1998a, Markham *et al.* 1998b, Olsson *et al.* 1998, Reuber *et al.* 1996a, Ryan *et al.* 1998).

However, glycosides make the flavonoids less reactive and thus less suitable for scavenging ROS. Because most flavonoids in plants are found in glycosylated or stable forms, they are not optimal for the function as antioxidant (Rice-Evans *et al.* 1997, Yamasaki 1997). Nevertheless, it seems that also flavonol glycosides play an important

role as antioxidant agent because many cases are found of larger increase of flavonols with an additional *ortho*-dihydroxyl group.

7. Hormonal controller. Flavonoids can alter the hormonal activity.

Indoleacetic acid (IAA) is a growth regulator of plants. Cofactors of the enzyme IAAoxidase are monohydroxy B-ring flavonoids such as kaempferol. This enzyme breaks down IAA. The dihydroxy B-rings, such as in quercetin, are inhibitors of IAAoxidase (McClure 1975, Harborne 1980). Both the mono- and dihydroxy B-rings inhibit the transport of auxin across the plasma membrane (Markham 1975, McClure 1975, Stafford 1991). A UV-B triggered alteration in concentrations of kaempferol and quercetin can lead to changed IAA activity and/or concentration. IAA can also be directly photodegraded by UV-B into photo-oxidation products, which in turn cause morphological changes (Jansen *et al.* 1998, Tevini & Teramura 1989)

It is known that auxin has an important regulatory function in apical dominance, cell division and cell elongation (causing stunting and dwarfing of plants) (Singh 1996). Altered morphology caused by UV-B, such as increased branching, reduced shoot length, small leaves (caused by lowered cell elongation and/or cell division) can be regulated indirectly via flavonoids and auxins (Jansen *et al.* 1998, Yalpani *et al.* 1994).

8. Other physiological functions. The leaf epicuticular exudates of *Dittrichia viscosa*, with numerous flavonoid aglycones, have an anti-transpiring function and are strongly allelopathic (Stephanou & Manetas 1997).

Functions of hydroxycinnamic acids.

The importance of hydroxycinnamic acids as UV-B induced screening agent is restricted because these compounds are mainly constitutive in the leaves. Moreover, the concentration of the soluble hydroxycinnamic acids in the leaf of barley on a molar base is lower than the flavonoid concentration (Liu *et al.* 1995). However, the hydroxycinnamic acids show in the UV-B range better absorption on a molar base in comparison with flavonoids (see Figure 4). All the same, hydroxycinnamic acids are important as constitutive UV-B absorbing compound.

Slightly increasing hydroxycinnamic acid concentrations in response to UV-B can lead to changing leaf morphology. Increased levels of ferulic acid in the lower epidermis enhance the number of carbohydrate-ferulic acid cross-links in the cell wall. This can reduce cell expansion (Liu *et al.* 1995).

Salicylic acid is also part of the phenylpropanoid pathway (Figure 2). This compound seems to have a special role in plants as a signalling molecule in the systemic acquired resistance (SAR) (Dixon & Paiva 1995, Yalpani & Raskin 1993). SAR is a defence

mechanism in plants, induced during pathogen infection, UV radiation and ozone. The defence is not restricted to the infection site but can also extend to tissues, unexposed to the stress factor. It is likely that SAR results from the activation of a combination of biochemical processes in plants (Yalpani & Raskin 1993, Yalpani *et al.* 1994). Since UV-B induced flavonoids are often restricted to the induction site, SAR probably does not play a role in flavonoid production. Apart from being a trigger of SAR, salicylic acid is the inducer of alternative oxidase activity and heat and odour production in the inflorescence of thermogenic species. Furthermore, salicylic acid can affect physiological processes such as the promotion of flowering and inhibition of stomatal closure (Yalpani & Raskin 1993).

Conclusions

In response to UV-B, enhanced levels of phenolics are found in the leaves. Also constitutive amounts of phenolics contribute to UV-B protection. Besides flavonoids, simple phenolics such as hydroxycinnamic acids and hydroxycinnamic acid esters are the main UV-B absorbing compounds.

The most important feature of phenolics in UV-B protection is their ability to absorb selectively the UV part from the solar radiation. In this way, UV damage will be reduced, whereas the photosynthesis is not influenced since phenolics hardly absorb in the PAR region of the spectrum.

The role of both types of compounds in UV-B protection can be very diverse. In most cases the simple phenolics mainly provide a constitutive shield for UV-B radiation, whereas the flavonoids also provide an inducible UV-B shield, depending on the "need" for UV-B absorption in the leaves. In some plant species, both types of compounds are induced by UV-B radiation. The mechanisms overlap to provide a multiple mechanism for UV-B protection. Depending on the (tissue) layer of the leaf, synthesis of hydroxycinnamic acid or flavonoid compounds can be induced. Both types of compounds can be combined in one UV-B absorbing pigment.

Many abiotic and biotic factors influence the quantity and quality of phenolics in plants. The concentrations and kinds of phenolics that are synthesized for UV-B protection vary with plant life form, plant species and even between cultivars. It seems that plant life forms with long-lived leaves and UV-B tolerant species and cultivars can produce a higher amount or more effective UV-B absorbing pigments in the epidermis. The amount of phenolics also shows spatial compartmentation. The accumulation of UV-B absorbing pigments is highest in the vacuole of the epidermal cells. The kind and amount of UV-B absorbing phenolics in plants differs with the developmental stage of plants and

leaves. Temporal effects can also influence the amount of phenolics in plants. Finally, the accumulation of flavonoids can be a UV-B dose dependent response.

There seems to be a specific increase of flavonoids with an additional *ortho*-dihydroxyl group. These groups do not alter the absorption characteristics of the flavonoids. There may be an extra UV-B protection because of the increased antioxidant activity of these compounds.

UV-B induced changes in plant morphology can be considered to be an indirect effect of changes in the phenylpropanoid pathway. The altered phenolics and IAA concentrations may cause thicker leaves, shorter stems and increased branching. Thicker leaves, including a thicker epidermal layer, may provide better UV-B absorption and may be beneficial to the plant in an enhanced UV-B environment.

Morphological changes in response to enhanced UV-B radiation under low ('PAR') and near ambient ('>>PAR') PAR levels.

Chapter 3

The response of *Vicia faba* to enhanced UV-B radiation under low and near-ambient PAR levels.

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Abstract

The effects of enhanced UV-B are often overestimated in greenhouse studies due to low levels of photosynthetically active radiation (PAR). For this reason, we studied effects of enhanced UV-B (12 kJ m^{-2} d^{-1}) at low and near-ambient PAR levels on young vegetative plants of *Vicia faba,* in the greenhouse. It was hypothesised that near-ambient PAR levels could reduce the negative UV-B effects on growth, due to higher amounts of UV-B absorbing compounds in the leaves and to morphological changes attenuating UV-B damage.

We found that effects of enhanced UV-B on the growth were not negative. An increase in biomass in response to enhanced UV-B at low and near-ambient PAR levels was observed The increase in biomass was related to increased branching which leads to a higher interception of PAR. Enhanced irradiance of both PAR and UV-B had similar photomorphogenic effects: thicker and smaller leaves and reduced plant height and internode length. Moreover, the concentration of UV-B absorbing compounds was increased. We conclude that in this study effects of enhanced UV-B were mainly photomorphogenic effects, which were also induced by radiation in the PAR region.

Abbreviations:
PAR: photosynthetic active radiation, RGR: relative growth rate, SLA: specific leaf area, LWR: leaf weight ratio, LAR: leaf area ratio, NAR: net assimilation rate IAA: indoleacetic acid

Introduction

During the last three decades, a decline in stratospheric ozone amounts has occurred. This decrease is ascribed to anthropogenically emitted CFCs (chlorofluorocarbons) and other ozone depleting chemicals reaching the stratosphere (Herman *et al.* 1996; Madronich *et al.* 1998). Also greenhouse gases, which cause cooling of the stratospheric ozone layer above the arctic, appear to be an indirect factor leading to ozone depletion (Shindell *et al.* 1998). As a result of this decline in stratospheric ozone concentration, plants receive increasing solar UV-B radiation levels (Caldwell & Flint 1994; Herman *et al.* 1996; Madronich *et al.* 1998).

Enhanced UV-B fluency rates can cause damaging effects in plants (Dumpert & Knacker 1985; Runeckles & Krupa 1994; Kim *et al.* 1998), for instance DNA damage by the formation of DNA dimers (Taylor *et al.* 1997). On the plant level, a reduced biomass production may occur. The growth reduction can be the result of a changed allocation of

biomass, increasing amounts of secondary compounds or morphological alterations which lead to lower photosynthetic productivity (Teramura *et al.* 1980; Caldwell *et al.* 1989; Fiscus & Booker 1995; Allen *et al.* 1998). Responses to UV-B include morphological alterations such as reduced leaf size, thicker leaves (Adamse & Britz 1992), reduced hypocotyl length (Kim *et al.* 1998) and curling and bronzing of leaves (Teramura *et al.* 1980; Visser *et al.* 1997a; Allen *et al.* 1998). These effects are more pronounced at lower PAR levels (Teramura *et al.* 1980; Warner & Caldwell 1983; Mirecki & Teramura 1984). Morphological UV-B effects could either be interpreted as damaging effects when they are caused by photodestructive- processes or as photomorphogenic responses mediated via photoreceptors (Barnes *et al.* 1996; Kim *et al.* 1998).

Photomorphogenic UV-B effects are observed at low UV-B doses, causing no damage (Tevini & Teramura 1989; Kim *et al.* 1998). Another UV-B response, mediated by a photoreceptor is the increase of UV-B absorbing compounds, such as flavonoids in the leaves, particularly in the epidermis (McClure 1975; Caldwell *et al.* 1989; Runeckles & Krupa 1994; chapter 2 of this thesis).

Some photomorphogenic effects and the production of flavonoids give mesophyll cells protection against UV-B radiation and thus have a role in adaptation to UV-B radiation (Teramura 1986; Ballaré *et al.* 1992; Barnes *et al.* 1996). When leaves become thicker, UV-B as well as PAR is absorbed in higher amounts in the leaves implying that leaf tissue is exposed to reduced levels of both UV-B and PAR (Adamse & Britz 1992; Ballaré *et al.* 1992). Also the increased amounts of flavonoids, which are produced in response to UV-B, may be favourable in a UV-B irradiated environment. Flavonoids absorb specifically in the UV region and not in the PAR region (e.g. McClure 1975; Ballaré *et al.* 1992). At higher PAR levels, the interaction between UV-B and PAR effects may lead to compensation of negative UV-B effects (Warner & Caldwell 1983; Cen & Bornman 1990; Adamse & Britz 1992; Ballaré *et al.* 1992). Firstly, radiation with a wavelength range between 300 and 500 nm is required for the activity of the enzyme DNA photolyase, repairing DNA dimers induced by UV-B (Jordan 1993; Taylor *et al.* 1997). Secondly, some UV-B effects such as reduced plant height, thicker leaves and enhanced concentrations of phenolics, which have a protective function against UV-B, are also observed in response to enhanced PAR levels (Teramura 1980; Cen & Bornman 1990; Ballaré *et al.* 1992). In most cases, PAR levels in the greenhouse and in climate chambers are lower than outside. Also, the light spectrum inside differs from the spectral composition of the light outside. Thus, when results from greenhouse experiments are extrapolated to the field situation, this may lead to an overestimation of UV-B effects on growth in the field (Kramer *et al.* 1992; Barnes *et al.* 1996; Rozema *et al.* 1997; Caldwell *et al.* 1998).

In the greenhouse and the climate room where environmental conditions can be standardised, we investigated the UV-B effects of *V. faba* with low PAR (250 μmol $m^{-2}s^{-1}$) and near-ambient PAR levels (600 μmol $m^{-2}s^{-1}$). We investigated whether greenhouse experiments overestimate the growth reduction by enhanced UV-B radiation due to low PAR levels. We measured growth, morphology and accumulation of UV-B absorbing compounds. It was hypothesised that enhanced UV-B radiation, in combination with near-ambient PAR levels, should lead to a less pronounced growth reduction, due to enhanced flavonoid production and an altered morphology (cf. Flint *et al.* 1985; Barnes *et al.* 1990; Visser et al.1997a,b; Tosserams *et al.* 2000).

Material and Methods

Growth conditions

The experiment was conducted in a ventilated greenhouse compartment with a 14 h photoperiod and day/night temperature regime of 22-26 °C / 14-16 °C. Relative humidity varied between 50% and 95 % depending on temperature. The experiment was conducted between September 23, and October 14, 1996. Daily global irradiance at Schiphol Airport, located about 10 km from the greenhouse, is shown in Figure 1.

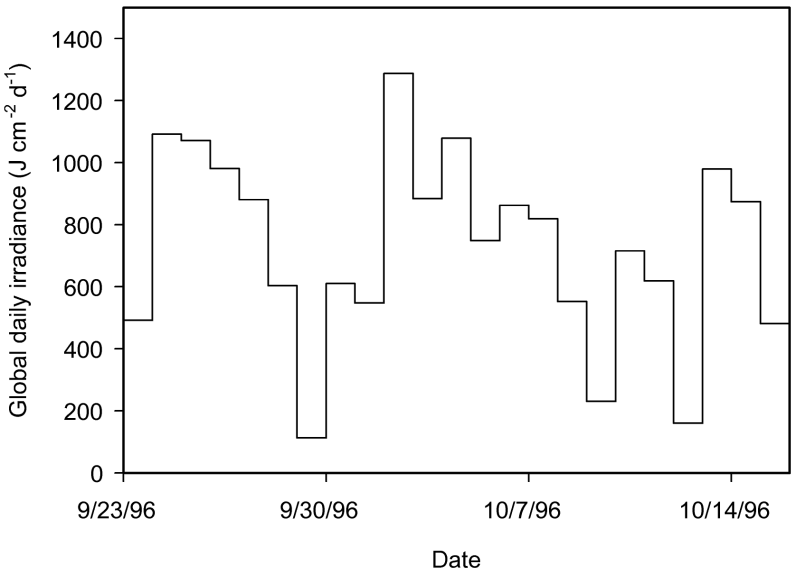

Figure 1. Global daily irradiance at Schiphol Airport, located about 10 km from the greenhouse, during the experiment.

Experimental set-up and light treatments

Two *Vicia faba* L. (cv. Minica) seeds were sown per pot (2.6 l) filled with a mixture of commercial potting soil (Jongkind BV, Aalsmeer, NL), 0.1 L of potting soil inoculated with *Rhizobium* bacteria, and 3 g L^{-1} of a controlled release fertiliser (Osmocote 13:13:13:3:2, N:P:K:Mg:Fe; Grace Sierra Int., Heerlen, NL). Ten days after sowing, thinning to one plant per pot took place.

Four treatments were used: *minus* (-UV-B) and *plus* UV-B (+UV-B) (Biologically Effective dose, $UV\text{-}B_{BE}$: 0 and 12 kJ m^{-2} d^{-1}, respectively) in combination with low (at least 250 µmol m^{-2} s^{-1}) (LL) and near-ambient (at least 600 µmol m^{-2} s^{-1}) (AL) additional PAR, indicated as -UV-B LL, -UV-B AL, +UV-B LL and +UV-B AL, respectively. Spectra of the PAR and UV-B treatments were measured with a double-monochromatic spectroradiometer (Optronic Model OL 752) at noon in the greenhouse (Figure 2).

Plants were divided at random over the treatments. The treatments started 14 days after sowing. Plants were rotated twice a week within the treatment plot. The treatment plots were rotated within the greenhouse once a week to avoid site effects. Per treatment, one experimental plot with 12 plants was used. Twenty days after starting the treatments, the plants were harvested for growth and analysis of UV-B absorbing compounds.

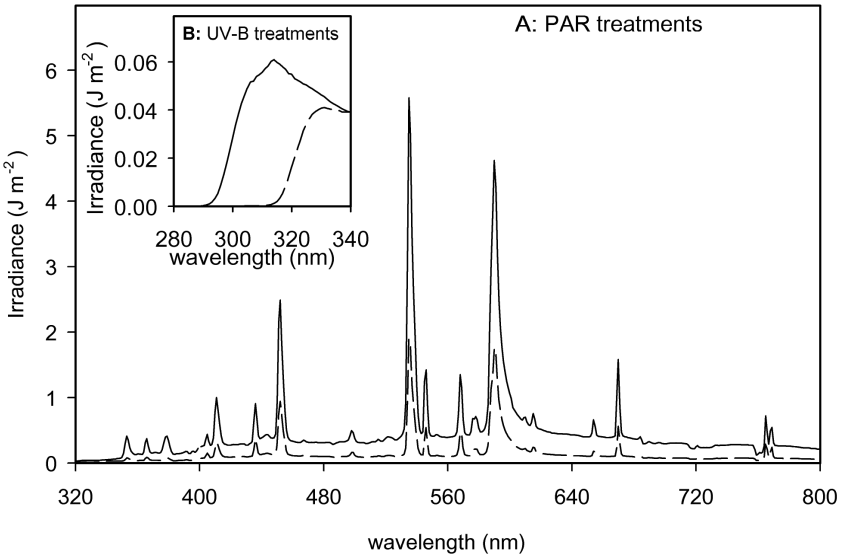

Figure 2. Examples of spectra taken around noon, measured with a spectroradiometer.
2A: Spectra of the low PAR (LL) (250 µmol m^{-2} s^{-1}, equivalent to 280 J cm^{-2} day^{-1}, dashed line) and near-ambient PAR (AL) (600 µmol m^{-2} s^{-1} equivalent to 673 J cm^{-2} day^{-1}, solid line).
2 B: Spectra of minus UV-B (-UV-B) (dashed line) and plus UV-B, 12 kJ UVBE m^{-2} day^{-1} (+UV-B) (solid line).

The average daily global irradiance was 736 J cm^{-2} d^{-1} (Figure 1). The near-ambient PAR treatment had a minimal flux of 600 µmol m^{-2} s^{-1} during 14 h per day (intensity of the lamps plus the minimal natural light intensity). This led to a daily irradiance dose of 673 J cm^{-2} d^{-1}. We converted the PAR flux to daily irradiance using a PAR spectrum measured in the greenhouse (Figure 2). For the low PAR level (250 µmol m^{-2} s^{-1}) we calculated a daily dose of 280 J cm^{-2} d^{-1}.

The PAR and UV-B doses were realised at canopy height and adjusted twice a week by adapting lamp levels above the canopy. The PAR dose was measured with a Li-185b quantum sensor (LI-COR Inc., Lincoln, NA, USA). Additional PAR was supplied 14 h a day by 400 W Philips HPI-T lamps. For the low PAR (LL) treatment, one lamp was used per plot. For the near-ambient PAR (AL) treatment, two HPT-I lamps were used per plot of which the lamp holders were covered inside with aluminium foil. Plants were exposed to UV-B by irradiation with two Philips 40W/12 lamps per plot, switched on from 10.00-16.00 h, which were wrapped in cellulose acetate foil (0.1 mm, Tamboer & Co. Chemie B. V., Haarlem, NL). This foil transmits the radiation above 290 nm (Figure 2). For the control UV-B treatment (*minus* UV-B), UV lamps were wrapped in polyester foil (Mylar, 0.13 mm), which excludes UV-B radiation below 313 nm (Figure 2). Mylar foil was renewed once a week and cellulose acetate foil twice a week. The UV-B dose was adjusted with a portable UV-X radiometer with a UV-X 31 sensor (San Gabriel, CA, USA). For this purpose, a calibration curve of the UV-X meter and the biologically active UV-B dose ($UV\text{-}B_{BE}$) was constructed. The $UV\text{-}B_{BE}$ dose was calculated by multiplying UV-B spectra measured with a spectroradiometer and weighting factors from the generalised plant action spectrum (Caldwell 1971), normalised at 300 nm. The $UV\text{-}B_{BE}$ dose of 12 kJ m^{-2} d^{-1} simulated 35 % ozone reduction at clear sky on 21 June in Amsterdam according to the model of Green *et al.* (1980).

Growth analysis, morphology and UV-B absorbing compounds

Plants were harvested after twenty days of the UV-B/ PAR treatment. The number of adventitious shoots and leaves per shoot were counted, and shoot length and leaf area (Licor 3100 area meter, Li-Cor Inc., Lincoln, USA) per shoot were measured. For determining biomass accumulation, fresh weight of leaves and stems of main and adventitious shoots were measured separately. Dry weight of all stems and leaves were measured after drying at 70 °C for 48 h. With the fresh weight to dry weight ratio, dry weight of leaves and stems per shoot was calculated. Dry weight of roots of the plants was determined after rinsing with water and drying at 70 °C for 48 h.

Internode length was calculated by dividing shoot length by number of leaves. Leaf

area per leaf was calculated by dividing leaf area of the whole shoot by number of leaves. Specific leaf area (SLA) per shoot is the ratio between leaf area and dry weight of the leaves per shoot. Leaf weight ratio (LWR) is the ratio of biomass between leaf and whole plant.

For the analysis of UV-B absorbing compounds, two leaf discs (1 cm^2) of young, just unfolded leaves of the main shoot were sampled. After measuring fresh weight of the samples, they were frozen in liquid nitrogen and stored at –20 °C for 14 days. UV-B absorbing pigments were extracted with 5 mL of a mixture of methanol: water: hydrochloric acid (79:20:1) at 90 °C for 90 min. The absorption spectrum of the extract was measured between 280 to 320 nm. Integration of this spectrum in the UV-B region was used as a relative concentration of UV-B absorbing compounds in the extracts.

Statistics

Data were statistically tested with SPSS software (SPSS Inc. version 8.0). Normality was tested with Shapiro–Wilk and homogeneity of variance was tested with Levene's test. Data of dry weight of the main shoot were transformed to their natural logarithm, to obtain homogeneity of variance. UV-B and PAR effects and interaction of UV-B * PAR was tested with two-way ANOVA followed by an LSD post-hoc test on the four treatments. Data of the number of shoots and number of leaves were not normally distributed and were tested non-parametrically by multiple comparisons with the Kruskal Wallis test (Zar 1984).

Results

Biomass accumulation

The total dry biomass of plants exposed to enhanced UV-B was higher than that of plants without exposure to UV-B ($p<0.001$), whereas there was no effect of PAR (Figure 3). A similar response was observed for the biomass of the roots and total aboveground dry weight. No UV-B effect on biomass accumulation of the main shoot was found, whereas at near-ambient PAR levels biomass accumulation of the main shoot decreased ($p=0.001$). Biomass accumulation of the first adventitious shoot increased in response to enhanced UV-B ($p<0.001$). A second and third adventitious shoot was developed in plants exposed to enhanced UV-B (Figure 3, Table 1).

We found an increased allocation of biomass to the roots in response to enhanced UV-B ($p<0.001$), reflected as a decrease in shoot to root ratio (Table 2). The leaf weight

Table 1: Number of shoots and number of leaves of main and first adventitious shoot of *V. faba*, in response to UV-B radiation under low and near-ambient PAR levels. Data are means ± SE with n=12. Different letters in columns indicate significant difference between treatments *(p*<0.05, LSD test). Treatments are *minus* UV-B (-UV-B) and *plus* UV-B: 12 kJ m^{-2} d^{-1} (+UV-B) in combination with low PAR, 250 µmol m^{-2} s^{-1} (LL) or near-ambient PAR, 600 µmol m^{-2} s^{-1} (AL).

Treatment	Number of shoots		Number of leaves of main shoot		Number of leaves of first adventitious shoot	
-UV-B LL	1.50 ± 0.19	a	14.3 ± 0.26	a	1.83 ± 0.87	a
-UV-B AL	1.92 ± 0.08	a	14.8 ± 0.25	a	5.75 ± 0.93	ab
+UV-B LL	3.08 ± 0.23	b	14.6 ± 0.36	a	8.33 ± 0.58	b
+UV-B AL	3.42 ± 0.19	b	14.7 ± 0.33	a	8.42 ± 0.57	b

Table 2. Shoot to root ratio (S/R) and Leaf weight ratio (LWR) of the total plant of *V. faba* , in response to UV-B radiation under low and near-ambient PAR levels. Treatments according to Table 1. Data are means ± SE with n=12. Different letters in columns indicate significant difference between treatments *(p*<0.05, LSD test).

Treatment	S/R		LWR	
-UV-B LL	3.0 ± 0.2	a	0.46 ± 0.02	a
-UV-B AL	3.1 ± 0.1	a	0.50 ± 0.01	b
+UV-B LL	2.4 ± 0.3	b	0.42 ± 0.01	c
+UV-B AL	2.2 ± 0.1	b	0.44 ± 0.01	ac

ratio (LWR) increased in response to near-ambient PAR levels *(p*=0.002) but decreased in response to enhanced UV-B radiation *(p*<0.001) (Table 2).

Plant architecture

More branching occurred in response to UV-B, while there was no PAR effect on branching (Table 1). The number of leaves per shoot can be seen as a measure for the developmental stage of the shoot. No difference in the number of leaves of the main shoot was found. Thus, there were no differences in developmental stage of the main shoot. The number of leaves of the first adventitious shoot increased in response to enhanced UV-B, which was comparable to the increased branching.

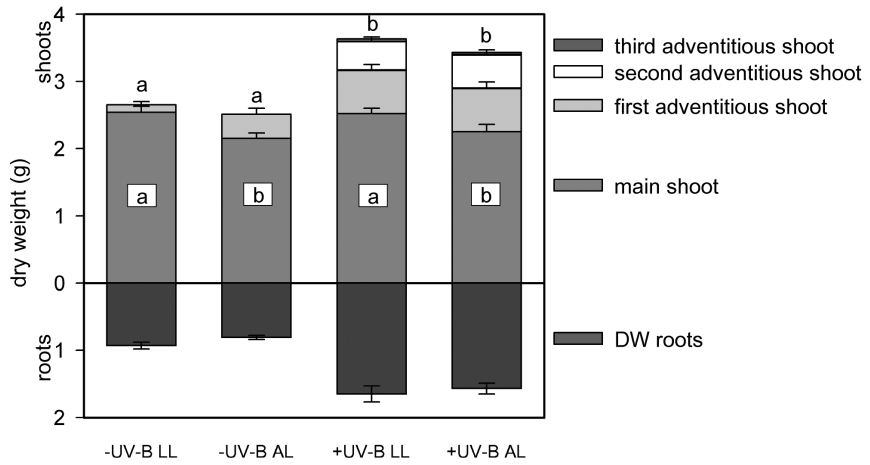

Figure 3. Dry weight of the roots and shoots of *Vicia faba* in response to UV-B radiation at low and near-ambient PAR levels. Treatments according to Table 1. Data are means ± SE per plant part (n=12). Different letters above and within bars indicate significant differences between treatments of dry weight of total plant and main shoot, respectively ($p<0.05$, LSD test).

The differences in length of the adventitious shoots could be caused by different developmental stages (less leaves) or by less elongation (shorter internodes). To estimate elongation of the internodes we calculated internode length. Enhanced UV-B radiation and PAR ($p<0.001$; Figure 4) reduced the internode length of the main shoot There was an interaction effect of the UV-B and PAR treatments on the main shoot ($p=0.003$) and a significant UV-B effect for the internode length of the first adventitious shoot ($p=0.034$). Plant height also decreased by high levels of PAR and UV-B radiation (data not shown).

Leaf morphology

Leaf size of the main shoot became smaller in response to near-ambient PAR ($p<0.001$) and UV-B radiation ($p=0.007$) (Figure 5). The combination of near-ambient PAR and enhanced UV-B was not additive. Leaf area of the first adventitious shoot was lower because of the younger developmental stage of the shoot. There was no treatment effect (UV-B or PAR) on the leaf size of the first adventitious shoot.

The specific leaf area (SLA) per shoot was calculated to estimate changes in leaf thickness. The SLA decreased in response to UV-B ($p<0.001$) and near-ambient PAR ($p<0.001$). Thus, leaves became thicker (Figure 6). The combination of UV-B and near-ambient PAR was not additive. There were no effects of UV-B and PAR on the SLA of the first adventitious shoot.

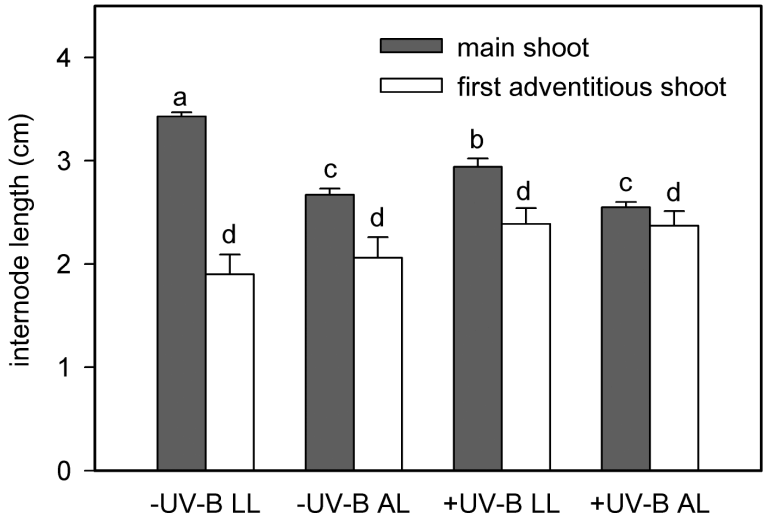

Figure 4. Internode length of *Vicia faba* in response to UV-B radiation at low and near-ambient PAR levels. Treatments according to Table 1. Data are means ± SE with n=12. Different letters above bars indicate significant differences between treatments of similar shoot type ($p<0.05$, LSD test).

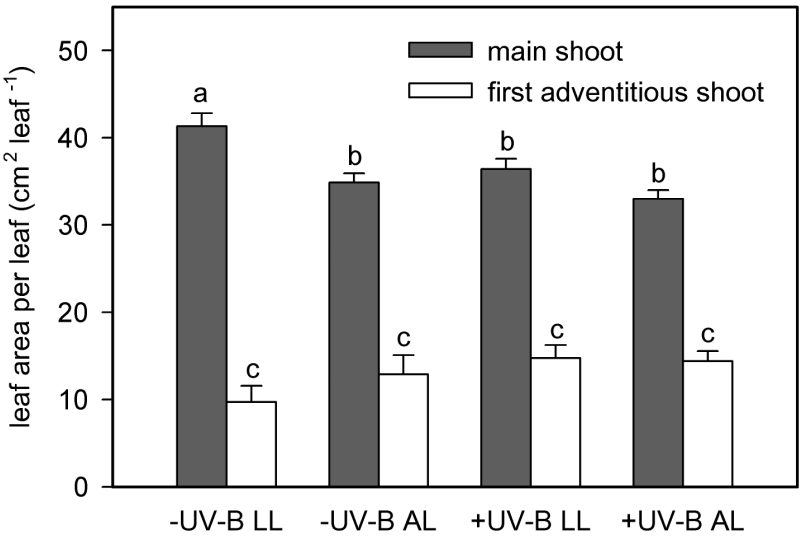

Figure 5. Leaf size of *Vicia faba* in response to UV-B radiation at low and near-ambient PAR levels. Treatments according to Table 1. Data are means ± SE with n=12. Different letters above bars indicate significant differences between treatments of similar shoot type ($p<0.05$, LSD test).

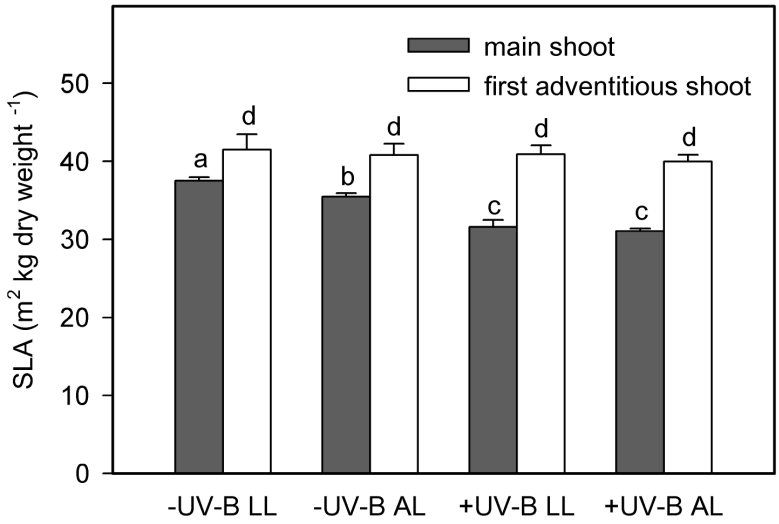

Figure 6. Specific Leaf Area (SLA) of *Vicia faba* in response to UV-B radiation at low and near-ambient PAR levels. Treatments according to Table 1. Data are means ± SE with n=12. Different letters above bars indicate significant differences between treatments of similar shoot type (*p<0.05*, LSD test).

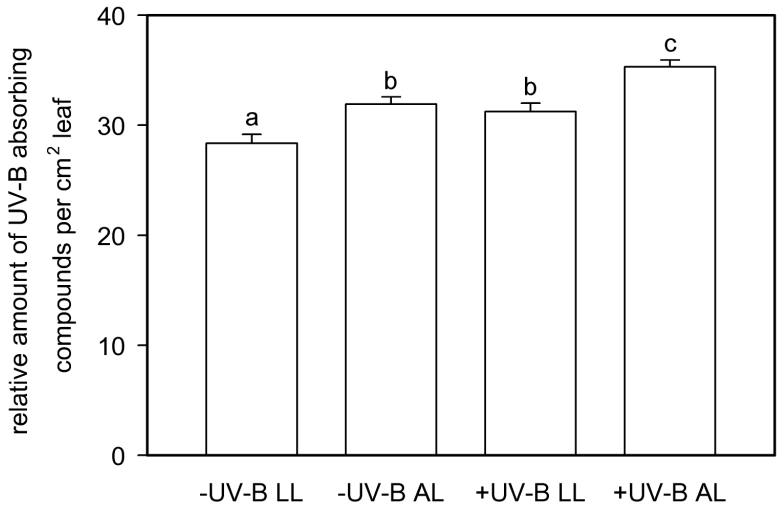

Figure 7. Relative amounts of UV-B absorbing compounds of *Vicia faba* in response to UV-B radiation at low and near-ambient PAR levels. Relative amounts are measured between 280 and 320 nm calculated on a leaf area base. Treatments according to Table 1. Data are means ± SE with n=12. Different letters above bars indicate significant differences between treatments (*p<0.05*, LSD test).

Pigments

Higher amounts of UV-B absorbing compounds were synthesised in leaves in response to UV-B (p<0.001) and PAR (p<0.001) (Figure 7). The accumulation of UV-B absorbing compounds in response to near-ambient PAR and enhanced UV-B radiation was additive (Figure 7).

Discussion

Growth

In contrast to our expectations, growth, measured as biomass accumulation, increased in response to enhanced UV-B radiation at both PAR levels (Figure 3). The increased biomass accumulation of the whole plant was caused by increased growth of adventitious shoots (Figure 3) bearing increased numbers of leaves (Table 1) so that more PAR could be captured and used for growth (Schmitt 1997). Furthermore, increased branching has led to relatively more young leaves with a higher photosynthetic rate. Some other experiments with *V. faba* showed no significant difference in biomass accumulation in response to UV-B, although morphology was altered (e.g. Barnes *et al.* 1990; Visser et al.1997b). However, Tosserams *et al.* (2000) found a growth reduction of the same cultivar (Minica) of *V. faba* in response to enhanced UV-B, at a PAR level of 250 µmol m^{-2} s^{-1} (day 24, 380 µmol CO_2 mol^{-1}, 0 and 10.6 kJ $UV\text{-}B_{BE}$ m^{-2} day^{-1}).

An explanation for these contrasting results may be differences in PAR intensity at the plant level at the time of the year in the greenhouse. The average global irradiance level outside during the UV-B treatment period of Tosserams *et al.* (2000) (end of December 1993/ January 1994) was 131 J cm^{-2} day^{-1} (KNMI 1993, 1994). During our experimental period (end of September/ October 1996), this dose was 736 J cm^{-2} day^{-1} (KNMI 1996). As the additional PAR levels were equal in both experiments, the total PAR level during the experiment of Tosserams *et al.* (2000) was much lower than during our experiment. The phenomenon that at lower PAR levels the UV-B growth responses were negative, which is not the case for higher PAR levels or outside situations, was also found in soybean, pea and many other crop species (Dumpert & Knacker 1985; Teramura & Sullivan 1987; Kramer *et al.* 1992; Deckmyn *et al.* 1994; Antonelli *et al.* 1997; Nogués *et al.* 1998; Allen *et al.* 1999).

Besides differences in PAR doses, spectral differences may help to explain contrasting growth responses to UV-B in our experiment and those of Tosserams *et al.* (2000). In addition, the plant density was higher in the experiments of Tosserams *et al.* (2000) resulting in more shaded spaces with higher UV-B to PAR ratio's in the canopy

(Grant 1997; Flint & Caldwell 1998; Deckmyn & Impens 1998). In a denser canopy, also the red to far-red ratio would be decreased and there would be a steeper drop in fluency rate of blue, red and far-red light (Ballaré *et al.* 1992). Altered light quality might also affect the morphology of plants (see below).

Deckmyn *et al.* (1994) ascribed the disappearance of negative UV-B effects at higher PAR levels to a higher PAR to UV-B ratio. However, it is unlikely that the ratio between UV-B and the whole PAR range is important, because there is not a general PAR receptor. Enhanced flavonoid concentration and photomorphogenic responses such as internode length, leaf size and leaf thickness, are responses induced by red, far-red, UV-A/blue and UV-B light. It is conceivable that the balance between red, far-red, blue/UV-A and UV-B is important for the final growth UV-B response, influenced by morphology and chemical content of the plants (Baraldi *et al.* 1998; Bornman 1999; chapter 2 of this thesis; Sullivan & Rozema 1999).

Photomorphogenic effects: morphology.

Increased branching of UV-B treated plants was related to increased biomass (Table 1). The internode length and leaf size of the main shoot were decreased and leaf thickness, measured as SLA, was increased by enhanced UV-B and PAR (Figures 4, 5 and 6). Plant height and leaf size were partly determined by internode and leaf elongation, respectively. These photomorphogenic characteristics were common characteristics of UV-B responses in *V. faba* and many other crop species (Dumpert & Knacker 1985; Cen & Bornman 1990; Barnes *et al.* 1996; Tosserams *et al.* 2000).

Many of these photomorphogenic UV-B effects were also initiated or modulated by other light qualities such as red, far-red, blue and UV-A radiation and high PAR (Chabot *et al.* 1979; Teramura 1980; Cen & Bornman 1990; Ballaré *et al.* 1992; Adamse *et al.* 1994). Red and far-red are perceived via phytochrome and blue/ UV-A light is primarily sensed by cryptochromes (Ballaré *et al.* 1992; Kraepiel & Miginiac 1997; Jenkins 1997). A possible explanation for the similarities and interactions between UV-B and red to far-red ratio on photomorphogenic effects may be that responses are mediated at least partly by phytochrome. Red, UV-A/blue as well as UV-B can transform the phytochrome activity (Young *et al.* 1992; Middleton & Teramura 1994; Jenkins 1997).

Based on this knowledge, we suggest that the photomorphogenic changes, which we found in response to UV-B and PAR, are possibly the result of the activity of cryptochrome, phytochrome, and other receptors. These receptors are regulated by UV-B, UV-A/blue and/or red and far-red light (Lingakumar & Kulandaivelu 1993; Barnes *et al.* 1996; Jenkins 1997; Kobzar *et al.* 1998). It is likely that the photomorphogenic

effects in response to UV-B, are not directly damaging UV-B effects (Ballaré *et al.* 1992; Barnes *et al.* 1996).

Increased branching and reduced plant height are characteristics of loss of apical dominance, which could be caused by reduction of indoleacetic acid (IAA) activity or auxin concentration (Schmitt 1997; Rozema *et al.* 2000). IAA is also involved in the light induced inhibition of plant cell elongation (Kraepiel & Miginiac 1997). It is suggested that IAA can be photodegraded directly by UV-B or that IAA activity can be reduced by interaction with quercetin flavonoids, which occur in the UV-B, irradiated *V. faba* leaves in enhanced amounts (Chapter 2 of this thesis). Ros and Tevini (1995) found that specific IAA photoproducts inhibit hypocotyl elongation in sunflower seedlings. All these data considered together, IAA might be involved in the UV-B transduction pathway leading to photomorphogenic responses. However, the role of IAA is still not clear in the light regulation of morphology (Kraepiel & Miginiac 1997).

UV-B absorbing compounds

Enhanced intensity of both PAR and UV-B led to higher concentrations of UV-B absorbing compounds. The effect of both radiation treatments is additive, thus enhanced PAR radiation gives additional UV-B protection (Figure 7). It is often found that the concentration of UV-B absorbing compounds is enhanced in leaves exposed to UV-B and enhanced PAR levels (Warner & Caldwell 1983; Cen & Bornman 1990; chapter 2 of this thesis).

For the induction of flavonoids, it seems that different light qualities (UV-B, UV-A/Blue and red/far-red) and thus different photoreceptors are working together (Ballaré *et al.* 1992; Beggs & Wellman 1994; Barnes *et al.* 1996, Jenkins 1997).

Damaging UV-B effects mediated by PAR

In addition to photomorphogenic effects, damage such as bronzing and curling of leaves has been found in Faba bean at a UV-B_{BE} dose of 10.6 kJ m^{-2} day^{-1} (Visser *et al.* 1997) and cucumber (Krizek *et al.* 1993). However, we did not find any visible damage on the leaves at a dose of 12 kJ m^{-2} day^{-1}. It seems that the PAR quality and quantity prevented leaves from damage by inducing UV-B protective responses. Also Adamse & Britz (1992), who used a UV-B_{BE} dose of 18 kJ m^{-2} day^{-1} in combination with a PAR dose of 1000 µmol m^{-2} s^{-1} did not find visible damage. Reduction of leaf size and internode length can also be considered UV-B damage, when this occurred by disturbance of cell division and elongation (Teramura *et al.* 1980; Mirecki & Teramura 1984). The UV-B fluency rate is determining whether damage or photomorphogenic effects take place

(Teramura *et al.* 1980; Kim *et al.* 1998). Maybe the threshold for occurrence of UV-B damage is dependent also on other light qualities than just UV-B.

The question was if near-ambient PAR (as compared to lower PAR levels) could reduce UV-B damage. As discussed above, PAR and UV-B can have the same photomorphogenic effects, which can be favourable to protect the mesophyll cells against deleterious UV-B radiation. However, for the direct growth and damaging effects, the effects of PAR and UV-B are not comparable. It is still incompletely understood which light quality and which mechanisms cause that PAR may ameliorate these damaging UV-B effects.

We conclude that, in the greenhouse, the effects of UV-B on growth of young vegetative *V. faba* plants are not always negative, even at "low" PAR levels. Photomorphogenic effects and an increase in UV-B absorbing compounds (Figure 7), are at least partly responsible for avoiding UV-B damage.

Acknowledgements

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Flavonoids were extracted from *Vicia faba* leaf discs and analysed with HPLC.

Chapter 4

Enhanced ultraviolet-B radiation increases the *in vivo* UV attenuance of *Vicia faba* leaves by induced synthesis of quercetin

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submitted

Abstract

The contribution of chemical and morphological leaf characteristics and leaf developmental stage to *in vivo* attenuance of ultraviolet (UV) radiation by *Vicia faba* leaves (cv. Minica and Pistache) was determined. Constitutive UV attenuance was high: more than 99.9% of the UV radiation (290-400 nm) was filtered out in non UV-B exposed plants. However, the UV transmittance was eight times lower in UV-B treated leaves. Leaf flavonoid concentrations and *in vivo* UV attenuance were highly correlated. Leaf thickness, non-soluble or cell wall bound phenolic compounds and kaempferol glycosides played a marginal role in the UV-B induced attenuance although they contributed largely to the constitutive UV screen. The UV-B induced attenuance increased during maturation of the leaves whereas the constitutive UV attenuance was already present in young leaves. UV-B induction of quercetin, which was the main factor determining the UV-B induced attenuance, was stronger in the UV-B tolerant cv. Minica than in the less UV-B tolerant cv. Pistache.

Abbreviations
Attenuance (A), photosynthetic active radiation (PAR), reactive oxygen species (ROS), specific leaf weight (SLW), Ultraviolet (UV)

Introduction

Doses of UV-B radiation (280-315 nm) reaching the earth's surface are enhanced, as a result of the breakdown of the ozone layer (McKenzie *et al.* 2003). This increase in short-waved radiation may have far-reaching consequences for plant morphology, physiology and chemistry (e.g. Rozema *et al.* 1997; Chapter 3 of this thesis).

At the molecular and cellular level, UV-B damage in plants consists of formation of DNA dimers, destruction of proteins, injury of membranes, damage to the photosynthetic apparatus, photo-oxidation of auxin and oxidative stress. The disruption of biomolecules results in altered physiological and growth processes; however, plants possess DNA repair mechanisms and antioxidants, which reduce sensitivity to UV-B (Greenberg *et al.* 1997).

Growth and physiology are also influenced by UV-B via photomorphogenic processes, which can be regulated by a photoreceptor (Greenberg *et al.* 1997). Common UV-B effects that diminish internal UV-B radiation levels are thicker leaves and accumulation of flavonoids, especially in the upper epidermis (Greenberg *et al.* 1997; Bornman *et al.* 1997; Chapter 2, 3 of this thesis).

It can be deduced from Beer's law for penetration of monochromatic light in a solution (absorbance), that the light intensity within and beneath leaves is determined by 1) the path length of the light within the leaf, 2) the chemical nature of leaf compounds and 3) the concentration of these leaf compounds. It should be noted that a leaf system is more complex than a solution (Day *et al.* 1993; Vogelmann, 1994). While the term absorbance describes attenuation in solutions, attenuance is used for *in vivo* leaf systems as a whole. The attenuance (A) is defined as the negative logarithm of the proportion of light, which is transmitted through the leaf (A= -log T). Both absorption and reflectance are included in the term attenuance (Terashima & Saeki, 1983, Vogelmann, 1994).

The path length of the light in leaves is dependent on leaf thickness, cell shape and refraction at different cell compartments causing internal scattering and reflection (Fukshansky *et al.* 1993; Gorton & Vogelmann, 1996). Compounds with specific UV-B absorbing properties are flavonoids and other phenolics like hydroxycinnamic acids (Bornman *et al.* 1997). The flavonoids in the upper layer of leaves function as a UV-B screen by filtering the UV-B radiation and not the PAR, which can reach the mesophyll nearly unattenuated. These morphological and chemical factors can jointly contribute to the constitutive and UV-B induced attenuance (Chapter 2 of this thesis).

This paper fills a gap in knowledge on UV-B adaptations and sensitivity in plants by coupling chemical and morphological leaf characteristics to the *in vivo* UV attenuance. For this purpose: 1) the *in vivo* UV attenuance in leaves of different developmental stages; 2) differential contributions of leaf thickness, non-soluble phenolic compounds and flavonoids to UV attenuance; 3) induction of specific flavonoids in response to UV-A and UV-B radiation were investigated.

The study was conducted with two cultivars of *Vicia faba* (cv. Pistache and Minica) different in growth response to UV-B (Chapter 1 of this thesis). Flavonoids attenuating UV-B radiation in *V. faba* leaves are glycosides of quercetin and kaempferol (Torck & Pinkas, 1992).

We hypothesised that the *in vivo* UV attenuance reflects UV-B sensitivity, which is changed during leaf development. In addition, we expected that both leaf thickness and flavonoids contribute to UV attenuance.

Material and Methods

Plant material and growth conditions

Experiments were performed in a greenhouse using two cultivars of *V. faba* L.: cv. Minica and cv. Pistache. An earlier experiment showed that growth of cv. Pistache was

reduced after 28 days of UV-B irradiation while growth of cv. Minica was unaffected (Chapter 1 of this thesis).

Seeds were sown as described in chapter 3 of this thesis. Fourteen days after sowing, treatments were started in a greenhouse with a 14 h photoperiod and a day/night temperature regime of 20-24 ºC/ 14-16 ºC. The greenhouse was equipped with an air-conditioning system to maintain nearly constant temperatures. Relative humidity varied between 50 % and 95 % depending on temperature and light period. During the 14 h photoperiod, PPFD was maintained to at least 250 μmol $m^{-2}s^{-1}$ (Philips HPI-T 400W) at canopy height, measured with a Li-185b quantum sensor (LI-COR Inc., Lincoln, NA, USA).

Experimental design

The experiment consisted of three treatments: only PAR (PAR), enhanced UV-A ("UV-A") and enhanced UV-A and UV-B ("UV-B"). The difference between the UV-B and the UV-A treatment can be considered as the pure UV-B effect. In the UV-B treatment plants were exposed to a biologically effective UV-B dose of 10 kJ m^{-2} d^{-1}, weighted with the generalised plant action spectrum (Caldwell, 1971). Two UV-B lamps (Philips 40W/12), wrapped in cellulose acetate foil (0.1 mm, Tamboer & Co. Chemie B.V., Haarlem, NL) and transparent for radiation above 290 nm, were installed above the plants. The "UV-A" treated plants were irradiated with the same lamps wrapped in polyester foil (Mylar, 0.13 mm), to filter out radiation below 313 nm but transmitting UV-A. With the PAR treatment, no UV lamps were lit above the plot, so that the plants only received PAR. The polyester foil was renewed once a week, the cellulose acetate foil twice a week. UV lamps were switched on from 10.00 to 16.00 h local time. Six plants were placed under one lamp system. During the experiment, plants were rotated twice a week to avoid site effects. The treatment blocks were rotated once a week within the greenhouse compartment to avoid site effects between treatments.

UV-B doses at canopy height were measured twice a week with a portable UV-B radiometer with a UV-X 31 sensor (San Gabriel, CA, USA) (cf. chapter 3 of this thesis) and adjusted to the intended level.

Leaves were harvested 21-24 d after start of the treatment. Six plants were used per treatment. The first (oldest leaf), third (mature leaf), fifth (mature leaf) and seventh (just unfolded) leaf of each plant were sampled. Leaf area (Licor 3100 area meter, Li-Cor inc., Lincoln, USA) and leaf fresh weight were determined. One leaf disc (3.1 cm^2) per sampled leaf was taken and used directly for the *in vivo* UV attenuance measurements. After extraction of the soluble flavonoids present in the leaf discs, the UV attenuance

measurement was repeated on the extracted, discoloured leaf discs, to estimate the attenuance caused by the non-soluble leaf compounds. The aqueous-methanol leaf extracts of the discs were prepared for flavonoid analysis by HPLC.

In vivo UV attenuance measurements

For the *in vivo* UV attenuance, leaf discs with a diameter of 2 cm (3.1 cm^2), were clamped in a black holder with an aperture size of 1cm x1.5 cm. The holder with the leaf disc was fixed to the sensor of the spectroradiometer and the irradiance was measured from 300 to 400 nm with a 2 nm interval (Optronic OL 752). A UV lamp (Philips 40 W/12) wrapped in fresh cellulose acetate foil was positioned 10 cm above the sensor as a source of ultraviolet radiation. Cellulose acetate was used to avoid exposure to UV-C radiation. In a similar way, the UV attenuance of methanol extracted leaf discs was measured. The extracted wet discs were patted dry carefully with tissue paper before attenuance measurements.

Adaptive scan duration was used to obtain measurements as accurately as possible. Therefore for a lower irradiance dose, a proportionally longer measuring time was used. Because of the relatively low UV doses below 300 nm, only an attenuance spectrum above 300 nm could be made.

To serve as a blank, the irradiance through the holder without a leaf disc was measured after every two samples measurement. Transmittance was calculated as a percentage of the irradiance of the leaf compared to the blank that was taken just before or after the measurement. Attenuance or apparent absorption, which has no unit, was calculated as the negative logarithm of the transmittance (Terashima & Saeki, 1983). Average attenuance of the leaf disc was calculated over the 300 to 400 nm intervals.

Flavonoid extraction and analysis

Flavonoids in leaf discs were extracted twice in 2.5 ml 90 % methanol. Discs in solution were shaken (60 rpm) for 9 and 15 h respectively in the dark at room temperature (20 ^{o}C). After that, discs were extracted twice in 2.5 mL 10 % methanol for 9 and 15 h under the same conditions. The four extracts were pooled. During the first extraction, a fixed amount of flavon was added as an internal standard.

Extracts were hydrolysed by adding 0.4 mL concentrated hydrochloric acid (36 w/w %) to 4 mL of centrifuged extract, according to Markham (1982). Extracts with added acid were boiled in closed Pyrex tubes for 1 h at 100 ºC. After hydrolysis, methanol was evaporated. Demineralised water (5 mL) was added to the samples together with 3 mL

ethylacetate. Tubes were shaken vigorously and the ethylacetate fraction containing the hydrolysed flavonoids was saved. Flavonoids in hydrolysates were extracted a second time using 1.5 mL ethylacetate. The pooled ethylacetate fractions were dried under reduced pressure in a speed-vac. Dried samples were stored at 4 ºC until HPLC analysis.

Before analysis, samples were dissolved in 200 μL of methanol with 0.1 % trifluoracetic acid (TFA) and filtered through a 0.2 μm nylon centrifuge filter (Costar Spin-X). The samples were run on a gradient of acetonitril (AN) and water with 1 % TFA at 60 ºC at a flow rate of 0.6 mL min^{-1}. The stepwise linear gradient started isocratically for 1 min with 8 % (AN), then 8 to 16 % (AN) for 29 min, 16% to 34 % (AN) for 7.75 min, 34 % to 81 % (AN) for 15.25 min, 81 % to 95% (AN) for 2 min, isocratically 95 % (AN) for 10 min, back to 8% (AN) in 2 min and finally kept on 8% (AN) for 13 min. The HPLC system was a Waters 2690 Alliance system equipped with two C-18 columns (3.9*150 mm) (Waters Nova Pak) and a C-18 guard column (Waters), with a Photo Diode Array detector (Waters 996).

For identification and quantification of the flavonoid aglycones in the extracts, calibration curves with commercial standards of flavon (Fluka), kaempferol (Roth) and quercetin (Sigma) were made. Identification of kaempferol and quercetin aglycones was done by comparing retention times of the samples with the purified aglycons. In addition, the UV spectra of the identified peaks were compared with the UV spectra of the purified aglycons. For quantification, absorption peaks of flavonoids at 254 nm were integrated.

Calculations of expected attenuance spectra based on flavonoid content

The influence of the flavonoids on the UV absorption ($A_{\lambda,\ flavonoids}$) was assessed using Beer's law. The following calculations were developed:

$$A_{\lambda,flavonoids} = A_{\lambda,quercetin} + A_{\lambda,kaempferol}$$

then

$$A_{\lambda,flavonoids} = \varepsilon_{\lambda,quercetin} * l * c_{quercetin} + \varepsilon_{\lambda,kaempferol} * l * c_{kaempferol}$$

then

$$A_{\lambda,flavonoids} = \varepsilon_{\lambda,quercetin} * SLW * W_{quercetin} + \varepsilon_{\lambda,kaempferol} * SLW * W_{kaempferol}$$

A_λ: Absorption at a specific wavelength λ

ε_λ: Molar absorptivity at a specific wavelength λ for the flavonoids quercetin or kaempferol (L mol^{-1} cm^{-1})

l: Path length of the light (cm)

c: Molar concentration of quercetin or kaempferol (mol L^{-1})

SLW: Specific leaf weight (g fw cm^{-2})

W: Amounts of quercetin or kaempferol in the leaf on a fresh leaf weight base (mmol g fw^{-1})

The molar absorptivity (ε_λ) was calculated every 2 nm for quercetin (Q) and kaempferol (K) with standard methanolic solutions of the aglycons in the UV range from 300 to 400 nm. For example, the molar absorptivity (L mol^{-1} cm^{-1}) at 300 nm was 7564 (Q) and 8384 (K) and at 370 nm 23124 (Q) and 18043 (K).

Average levels of quercetin and kaempferol per treatment and per leaf age were used together with the SLW for the estimation of the absorption graph based on flavonoid levels within the leaf.

Based on the flavonoid concentrations measured in the extracts and the extinction coefficients, an absorption spectrum was calculated for comparison with the *in vivo* attenuance spectrum of the fresh leaves.

Statistics

Data were tested statistically using SPSS software (SPSS Inc. version 8.0). Normality was tested with Shapiro-Wilk and homogeneity of variance was tested with Levene's test. Attenuance of extracted leaf was arcsine square-root transformed, to obtain homogeneity of variance.

A two way ANOVA with repeated measures was performed. The four leaf numbers (1, 3, 5 and 7) of one plant were taken as a repeating factor. Variance factors were cultivar and treatment. UV-A and UV-B effects were analysed, respectively, by paired comparisons of the "PAR" (without additional UV light) with "UV-A" (additional UV-A light) and "UV-B" treatments.

Spearman's correlations coefficients (Spearman's ρ) between flavonoid amounts (on a leaf area base) and $UV_{300-400}$-attenuance $_{[\text{fresh - extracted leaves}]}$ and between SLW and attenuance $_{[\text{extracted leaves}]}$ were also calculated per group (leaf number*cv) (Sokal & Rohlf 1995).

Results

Attenuance

The *in vivo* UV attenuance of fresh leaves of different ages of two cultivars of *V. faba* (cv. Minica and Pistache) was increased in response to UV-B (Figure 1) ($p < 0.001$ paired comparisons). Average attenuance in the UV range of UV-A treatment was 2.7-3.1, depending on leaf age and cultivar. This means that the UV transmittance was 0.08-0.2 %. UV-B treated mature leaves had an average attenuance of 3.6-3.9, which relates to a UV transmittance of 0.013-0.025 %. The UV transmittance was a factor 8 ($=10^{0.9}$, average difference in attenuance was 0.9) lower in UV-B treated leaves than in UV-A and PAR treated plants (Figure 1). The UV-A effect on *in vivo* UV attenuance was smaller than the UV-B effect, but also significant ($p=0.003$). The total *in vivo* UV attenuance is composed of the constitutive and UV-B induced attenuance.

The attenuance of extracted leaves treated with UV-A was lower than the PAR and UV-B treatment ($p=0.001$), whereas the difference between the PAR and UV-B treatment was not significant ($p=0.107$). Average attenuance values were between 0.7-1.0 (Figure 1). Therefore, after removal of the soluble compounds, the transmittance through the leaves was increased 100 to 1000 fold. The high increment in UV attenuance of fresh mature leaves occurring after UV-B treatment (UV-B induced attenuance) disappeared after methanolic extraction (compare the difference between UV-A and UV-B treatment of fresh leaves with this difference of extracted leaves).

There was a significant interaction between the treatment effect and leaf age effect ($p<0.001$). UV-B induced attenuance increased with leaf age, whereas constitutive attenuance decreased with maturation of the leaves (Figure 1).

Flavonoids

The aglycone components kaempferol and quercetin were found in the hydrolysed methanolic extracts. The concentration of quercetin increased from nearly zero for the PAR and UV-A treatment, to very high levels in UV-B treated leaves (Figure 2). In mature leaves, these levels were comparable to the kaempferol levels in UV-B treated leaves. ANOVA showed a significant UV-B treatment effect for quercetin ($p<0.001$) and no UV-B effect on kaempferol levels ($p= 0.078$). The concentrations of quercetin in UV-B treated leaves were higher in cv. Minica than in cv. Pistache when comparing leaves of equal developmental stage, except for just unfolded leaves. The UV-A effects on leaf quercetin concentration were less pronounced than the UV-B effects. An enhancement of the

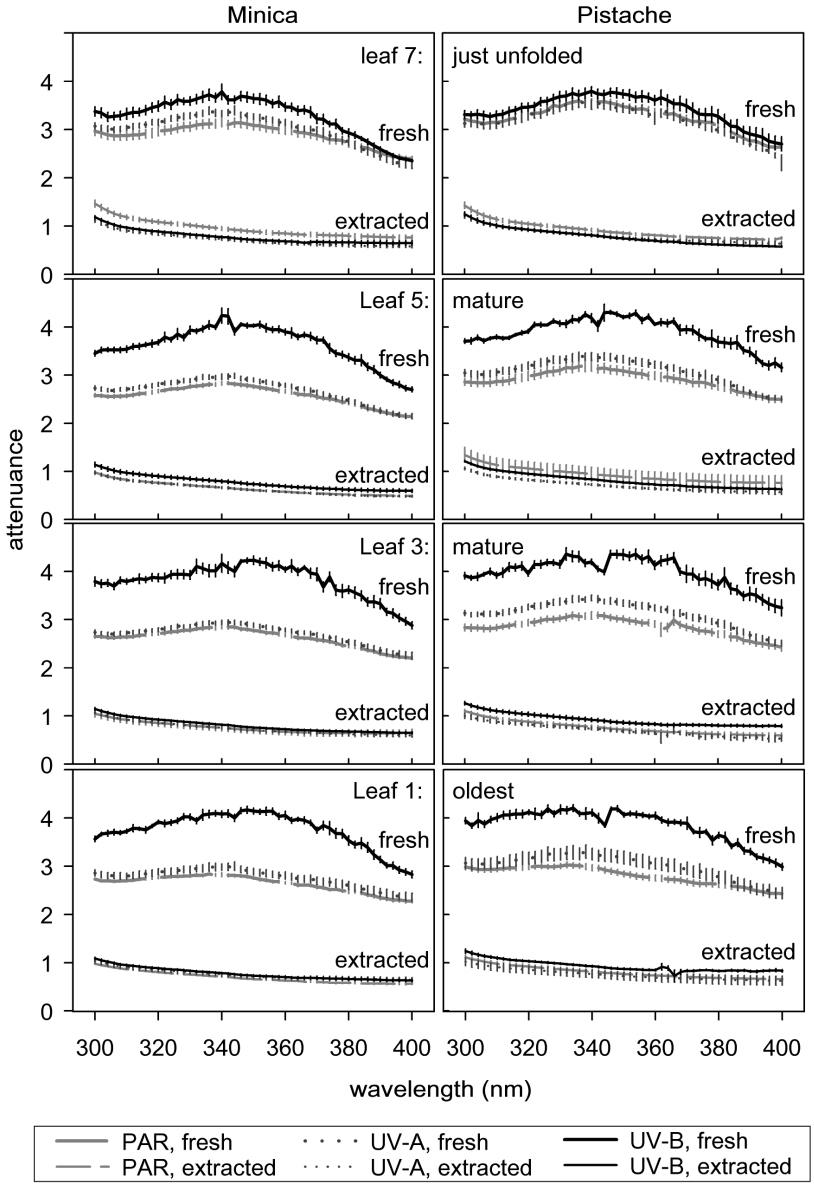

Figure 1. Attenuance spectra of fresh (upper three thick lines) and methanol extracted leaf discs (lower three fine lines) of *V. faba* cv. Minica (left graphs) and cv. Pistache (right graphs) in response to UV-B radiation. Data are means per 2 nm ± SE (n=6). Treatments are PAR radiation only (PAR: dashed lines), additional UV-A radiation (UV-A: dotted lines), and UV-B plus UV-A radiation (10 kJ UV-B_{BE} m^{-2} day^{-1}) (UV-B: solid lines). From top to bottom: leaf 7 (just unfolded), 5 (mature), 3 (mature) and 1 (oldest).

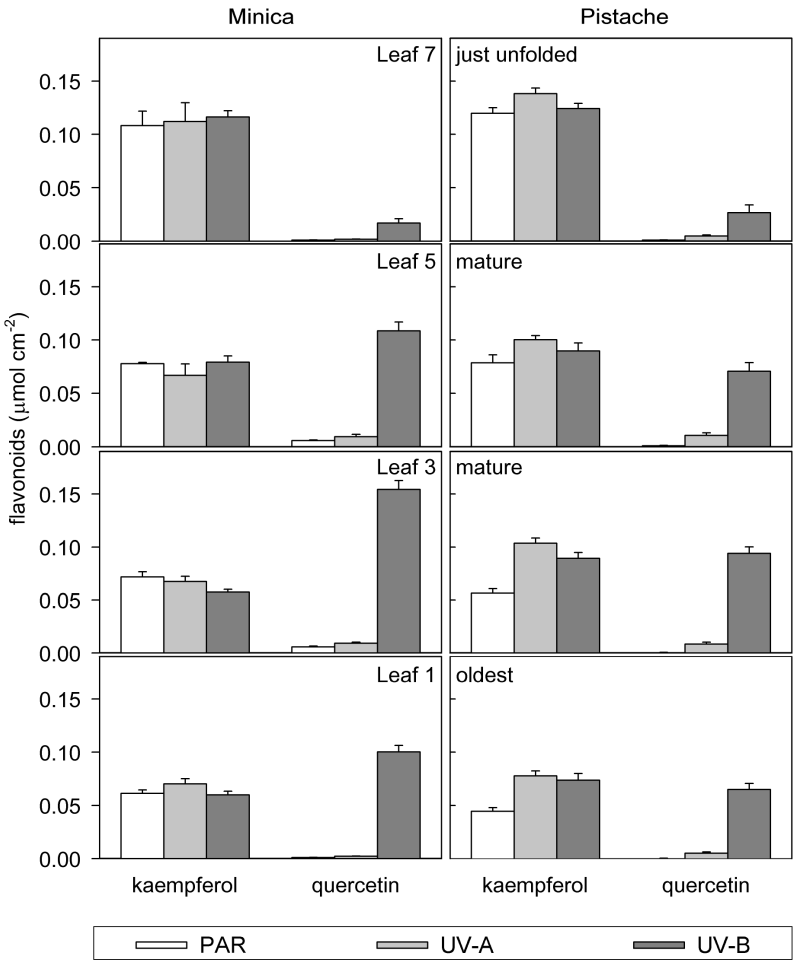

Figure 2. Amounts of kaempferol and quercetin per unit leaf area, in extracts of *V. faba* leaves cv. Minica (left graphs) and cv. Pistache (right graphs) in response to UV-B radiation. Data are means ± SE (n=6). Treatments: see Figure 1.

maturation of the leaves, the quercetin concentration was enhanced in UV-B treated leaves, except for the oldest leaves (leaf 1). For kaempferol, high concentrations in young leaves and a decrease in concentration with maturation occurred (Figure 2).

Kaempferol amounts calculated per whole leaf (concentration cm^{-2} * leaf area) showed that the final amount of kaempferol was already synthesised in leaf 7 (just unfolded) and that this amount did not change very much in leaf 3 and 5. In contrast, the amount of quercetin per whole leaf increased with maturation in UV-B treated plants (results not shown). The total flavonoid concentration in UV-B treated leaves increased with maturation of the leaves, and decreased in oldest leaves (leaf 1).

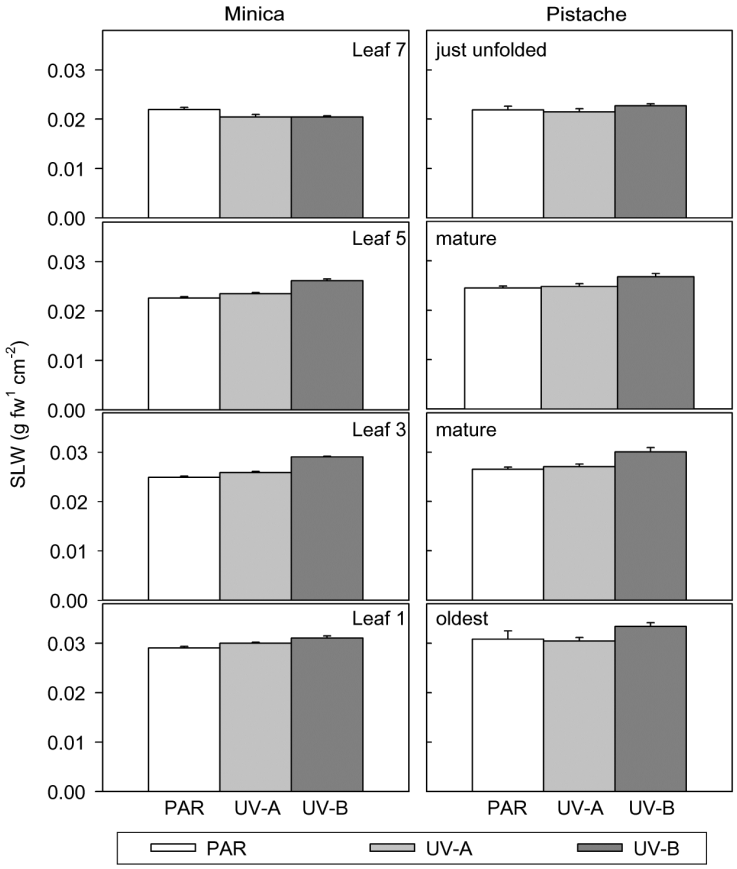

Figure 3. Specific leaf weight (SLW) of *V. faba* leaves cv. Minica (left graphs) and cv. Pistache (right graphs) in response to UV-B radiation. Data are means ± SE (n=6). Treatments: see Figure 1.

Specific leaf weight

The specific leaf weight was used as a measure for leaf thickness although, strictly speaking, SLW is defined by both leaf thickness and density. SLW was lowest in just unfolded leaves (leaf 7). Therefore, younger leaves were thinner than mature leaves. Paired comparisons showed that elevated UV-B resulted in increased leaf thickness for both cultivars in mature and old leaves (Figure 3).

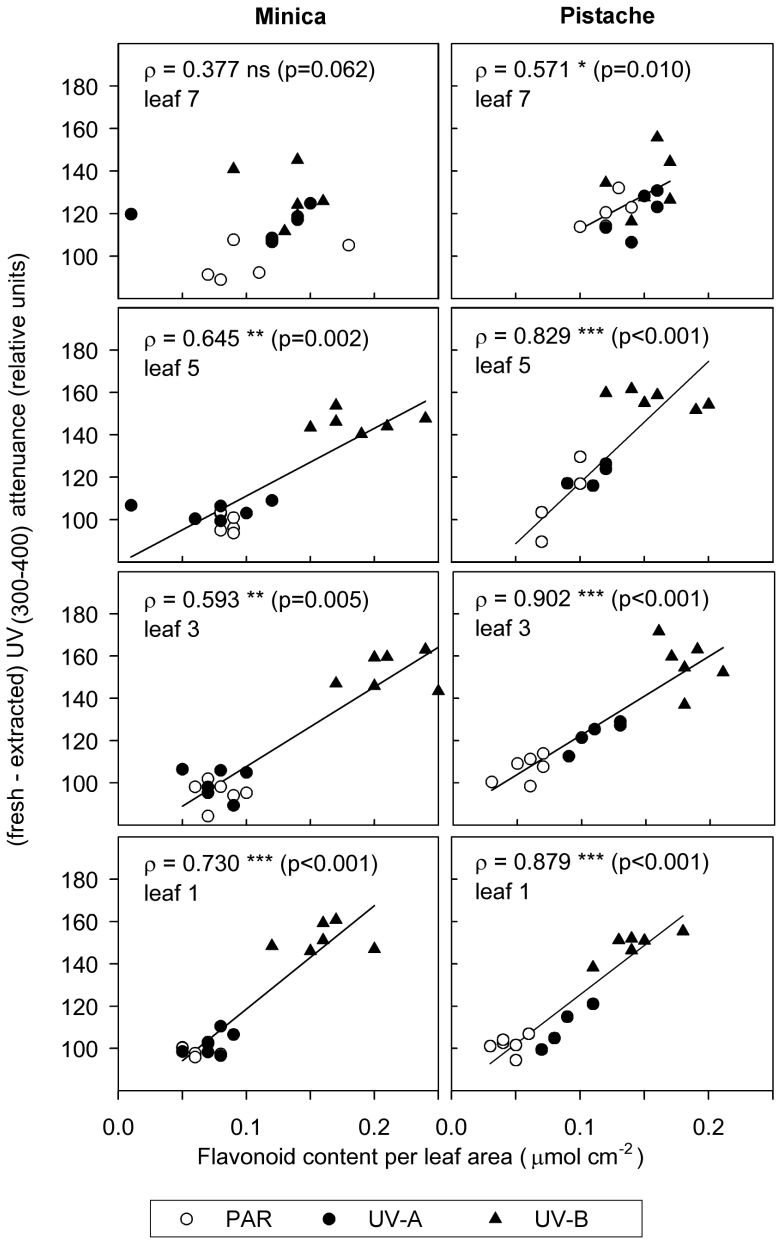

Figure 4. Spearman's correlations of integrated difference between attenuance of fresh and extracted leaf discs versus flavonoid amounts on a leaf area base. Regression lines are depicted per leaf stage and per cultivar of *V. faba* (cv. Minica left, cv. Pistache right). Treatments: see Figure 1.

Relations between flavonoids and *in vivo* attenuance

To assess the influence of leaf flavonoid levels on the *in vivo* UV attenuance, Spearman's correlation coefficients between flavonoids (on a leaf area base) and the *in vivo* attenuance of fresh leaves minus the spectrum of extracted leaves were calculated. Correlations between flavonoids and attenuance were significant in mature leaves *(p*≤0.005; Figure 4). Correlations between attenuance of the extracted leaf and specific leaf weight (SLW) were not significant (results not shown).

The contribution of the flavonoids to the *in vivo* UV attenuance was estimated quantitatively. A peak in UV attenuance in the UV-A range in the calculated spectrum was found, which was absent in the observed attenuance spectrum (Figure 5). This maximum in the UV-A range was also found in the spectra of the flavonoids (B. Meijkamp, unpublished results). Furthermore, the calculated UV attenuance was lower than the observed attenuance in the UV-B range, whereas in the UV-A range, the calculated attenuance exceeded the observed attenuance of UV-B treated samples.

Discussion

The total level of *in vivo* UV attenuance

UV attenuance measurements in this paper indicated a very low transparency of the whole leaf for UV radiation. A high UV attenuance of 3-4 in *V. faba* leaves (cv. Minica and Pistache) was found in all developmental stages and treatments (Figure 1). Considering the logarithmic scale, this implies that at most 0.1 % of the UV radiation was transmitted. As a result, penetration of the harmful UV-B radiation was effectively reduced in a *V. faba* leaf, although leaves of other plant life forms may have still higher attenuance levels (Day *et al.* 1992).

The *in vivo* attenuance of leaves of only the upper part of the UV-B range (300-320 nm) is presented although the lower region of the UV-B spectrum (280-300 nm) is also important in causing UV-B damage regarding the general plant action spectrum (Caldwell, 1971). However, the UV-B dosage between 280 and 300 nm during attenuance measurements was too low to get reliable irradiance data. The UV-B level between 280 and 300 nm during the measurements was not enhanced because the intensity would become irrelevantly high and could cause acute damaging effects during the measurements.

The transparency for UV radiation was not homogeneously distributed over the leaf area (Day *et al.* 1993). For example, at periclinal cell walls and beneath bundle sheath

extensions, the transparency for UV radiation was higher, especially in herbaceous leaves like *V. faba* (Gorton & Vogelmann, 1996; Day *et al.* 1993).

Reported epidermal attenuance was around 0.3-1.0 in UV-B irradiated *V. faba* leaves and other herbaceous dicots and therefore far lower than that of the whole leaf (Flint *et al.* 1985; Barnes *et al.* 2000). Therefore, in addition to the upper epidermal layer, attenuance of UV-B radiation also occurred in the mesophyll and lower epidermis. This implies that the mesophyll and lower epidermis should not be neglected when studying UV screens.

The *in vivo* UV attenuance of both cultivars differing in UV-B sensitivity was comparable. In contrast, the UV-B induced attenuance (difference between UV-A and UV-B treatment) of the less sensitive cv. Minica was higher than that of the sensitive cv. Pistache (Figure 1). Moreover, in young, more UV-B sensitive leaves (Day *et al.* 1996), the UV-B induced attenuance was low and the constitutive attenuance was high (Figure 1). Therefore, the UV-B induced attenuance may be probably a better indicator for UV-B sensitivity than the total *in vivo* UV attenuance.

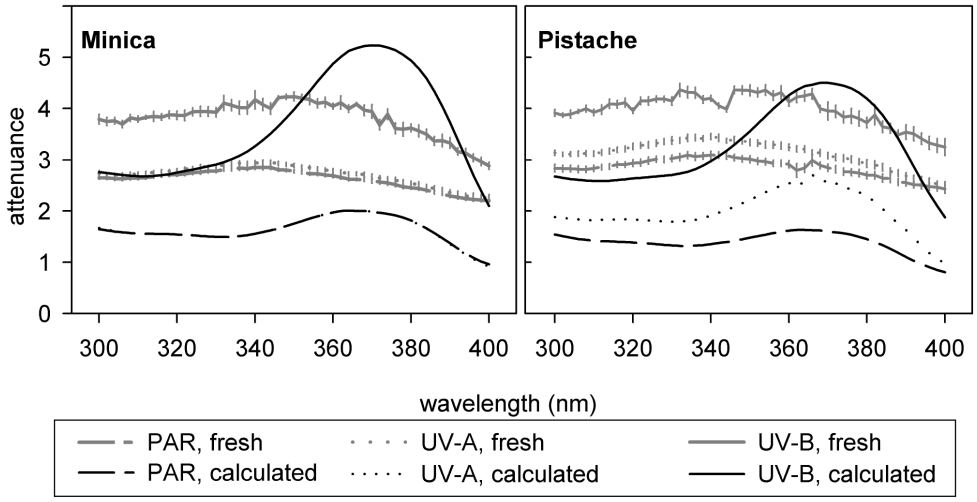

Figure 5. Calculated attenuance spectra of leaf 3, based on attenuance of extracted leaf discs and flavonoids (thin black lines) and as a comparison, *in vivo* attenuance spectra of fresh (thick grey lines) leaf discs (also shown in Figure 1) of *V. faba* cv. Minica (left graphs) and cv. Pistache (right graphs). Treatments: see Figure 1.

Constitutive role of leaf thickness and non-soluble or cell wall bound compounds in UV attenuance

An increment in SLW was observed in mature leaves in response to UV-B (Figure 3), indicating thickening of leaves. Strictly speaking, SLW is defined by both leaf thickness and density but leaf density is not altered in response to UV-B (B. Meijkamp, unpublished results). Increased leaf thickness in response to UV-B has been reported earlier and is regarded as providing protection to UV-B (e.g. Visser *et al.* 1997; chapter 3 of this thesis). However UV-B increased leaf thickness and non-soluble or cell wall bound phenolic compounds do not play an important role in the UV-B induced attenuance since all UV-B induced attenuance disappeared after extraction of the leaf discs (Figure 1). This is confirmed by the low and not significant correlations between SLW and *in vivo* UV attenuance in these experiments and those of Visser *et al.* (1997). Nevertheless, leaf thickness and wall bound compounds contribute largely to the constitutive UV screen, as indicated by the UV attenuance of extracted discs (around 0.8, Figure 1). These values are relatively low in comparison with those in the fresh leaf tissues but even then, only 16 % ($10^{-0.8}$) of the UV light is transmitted through the extracted leaf. In comparison with other plant life form groups with thicker leaves, the constitutive attenuance, caused by non-soluble phenolic compounds and leaf thickness is small in herbaceous species such as *V. faba* (Robberecht & Caldwell, 1986).

Contribution of flavonoids to the UV screen in leaves

In leaves of *V. faba* an increase especially in quercetin flavonoids in response to UV-B was found (figure 2). It was shown that soluble compounds like flavonoids contribute to UV attenuance in mature leaves of *V. faba*. *In vivo* attenuance of fresh minus extracted leaves was highly correlated with flavonoids amounts within the leaf (Figure 4). Also in other plant species, flavonoids are considered to be UV-B absorbing compounds (Robberecht & Caldwell, 1986; chapter 2 of this thesis).

By comparing the observed *in vivo* UV attenuance spectrum with the calculated spectrum, deviations in shape and attenuance level were found. The absolute value of observed attenuance exceeded the calculated attenuance in the UV-B region (Figure 5). The simplifications of the model, enumerated in Table 1, could explain these deviations in total attenuance.

In spite of the deviations between observed and calculated attenuance spectra, the UV-B induced attenuance (that is the difference in attenuance between UV-A and UV-B treated leaves) in the UV-B region is comparable in magnitude in calculated and observed

attenuance spectra (Figure 5). It has been established that these simplifications have small effects on the UV-B induced attenuance. Only the non-homogeneous distribution of flavonoids could have bigger effects (Chapter 5 of this thesis).

We therefore assume that the UV-B induced *in vivo* attenuance in *V. faba* leaves can be explained by the increase of flavonoids and especially quercetin (Figure 2, 5). UV-B induced quercetin concentration in the leaves of some cultivars of soybean and *Brassica* also provide a good UV-B protection (Bornman *et al.* 1997, Mazza *et al.* 2000). The less UV-B sensitive cv. Minica had a higher level of UV-B induced flavonoids (quercetin glycosides) than the more sensitive cv. Pistache (Figure 2). Therefore, we consider the amount of quercetin, which determines the UV-B induced attenuance, as a good indicator of UV-B sensitivity.

It is very well possible that in *V. faba* not only the total concentration of flavonoids, but also the concentration of UV-B induced quercetin or ratios between the flavonoid types (e.g. quercetin to kaempferol ratio) relate to UV-B sensitivity (Figure 2), as is the case in *Petunia* and *Brassica napus* (Ryan *et al.* 1998; Olsson *et al.* 1999).

The phenomenon that monohydroxy B-ring flavonoids such as kaempferol are constitutive whereas flavonoids with an additional hydroxyl group of the B-ring such as quercetin are UV-B induced (Figure 2) was also observed in other dicots (Bornman *et al.* 1997; Wilson *et al.* 1998; Ryan *et al.* 1998; Olsson *et al.* 1999). Although kaempferol and quercetin have a similar UV-B absorption spectrum and are therefore equally effective in UV-B screening, they differ in their ability to scavenge reactive oxygen species (ROS) (Wilson *et al.* 1998). Quercetin flavonoids are more effective in scavenging ROS than kaempferol flavonoids, which make quercetin more effective in protection against UV-B (Hideg *et al.* 1997; Greenberg *et al.* 1997).

We conclude that the UV-B induced protection (measured as an increase in UV attenuance) is mainly caused by increased concentrations of quercetin glycosides. Changes in SLW (indicator for leaf thickness) contribute only marginally to UV-B induced protection. The constitutive UV protection is caused by leaf thickness, cell wall bound phenolic compounds, flavonoid glycosides of kaempferol and quercetin and other UV-absorbing compounds (such as hydroxycinnamic acids). The constitutive attenuance is an important part of the UV screen because it is still a part of the UV screen after UV-B irradiation. However, UV-B tolerance in *V. faba* is probably linked with UV-B induced quercetin, since quercetin has a protective role both by absorbing UV-B radiation and by scavenging UV-B induced ROS.

Table 1. Simplifications in the model used to calculate contribution of flavonoids to *in vivo* UV attenuance (presented in Figure 5).

	Neglected factors in the model	References
1	The non-homogeneous distribution of flavonoids in the leaf tissue and in the cells.	chapter 5 of this thesis
2	Chlorophylls which have also UV-B absorbing properties. Chlorophyll concentrations are enhanced in response to UV-B in *V. faba.*	Visser *et al.* 1997
3	Hydroxycinnamic acids, which play a role in constitutive UV attenuance in most plant species.	Bornman *et al.* 1997
4	Multiple UV scattering, which elongates the path length. The path length of the light is assumed to be equal to the leaf thickness.	Terashima & Saeki, 1983, Fukshansky *et al.* 1993, Vogelmann, 1994
5	Flavonoid glycosides and not aglycons accumulate in *V. faba* leaves.	Torck & Pinkas, 1992 Markham, 1982
6	Reflectance by the cuticle.	(Robberecht & Caldwell, 1986; González *et al.* 1996).
7	Fluorescence of cell components through which UV light is converted to blue light.	Bilger *et al.* 2000

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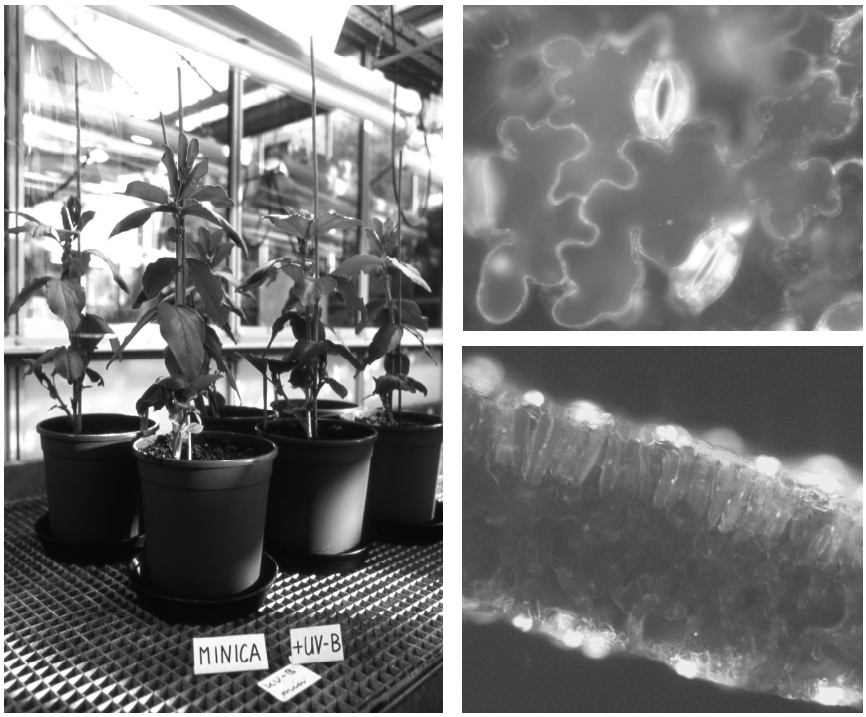

The epidermal layer with guard cells of *Vicia faba* leaves, which contains enhanced amounts of flavonoids after UV-B irradiation.

Chapter 5

Functional localisation of protective flavonoids in leaves of *Vicia faba* induced by UV-B radiation.

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Wilfried H.O. Ernst, Jelte Rozema

submitted

Abstract

Flavonoids in leaves of *Vicia faba* play a role in UV-B protection by filtering the harmful UV-B radiation. The localisation of these flavonoids is crucial for the UV-B screening effectiveness. We investigated the localisation of constitutive kaempferol flavonoids and UV-B induced quercetin flavonoids in *V. faba* leaves with fluorescence microscopic images. Naturstoffreagenz A and ammonia were used as fluorescence inducing agents. These qualitative investigations were supported with quantitative tissue-specific analysis of these flavonoids.

Enhanced UV-B radiation resulted in a fourfold increase of quercetin flavonoid concentrations in the leaves, which only accumulated in the adaxial epidermis. Small amounts of constitutive (not UV-B induced) quercetin were observed in the mesophyll and the abaxial epidermis. Kaempferol flavonoids, located in the epidermis and the mesophyll, were always found to be constitutive. As a result, there was a flavonoid concentration gradient from the adaxial to the abaxial epidermis with highest levels in the adaxial epidermis and lowest in the spongy parenchyma. There was no visibly altered localisation observed in response to UV-A radiation. In addition to these patterns, spots with high concentrations of flavonoids were observed around the nucleus and chloroplasts of palisade parenchyma. This could function as a microscreen providing additional UV-B protection to these UV-B sensitive organelles. Also the guard cells contained higher quercetin concentrations than their surrounding epidermal cells.

The localisation of the UV-B induced quercetin especially in the adaxial epidermis and constitutive flavonoids as microscreens around nucleus and chloroplasts indicate that UV-B filtering is the main function of these flavonoids. However, the exclusive induction of quercetin indicates that other (UV-B protective) functions such as reactive oxygen species scavenging can be assigned to the flavonoids.

Abbreviations
Naturstoffreagenz A (NA), reactive oxygen species (ROS), Ultraviolet (UV)

Introduction

Due to the reduction in stratospheric ozone, UV-B levels on earth are enhanced (Rozema *et al.* 2002). Although the anthropogenic emission of CFCs is highly reduced, recovery of the ozone layer is still not proven. Other factors i.e. global warming appear to delay the recovery of the ozone layer by affecting stratospheric temperatures (McKenzie *et al.* 2003).

Even through an undamaged ozone layer, part of the UV-B radiation emitted by the sun is reaching the earth's surface (Rozema *et al.* 2002). UV-B radiation can cause damage to DNA and photosystems and enhances reactive oxygen species (ROS) formation, which in turn leads to oxidative stress and membrane damage (Greenberg *et al.* 1997). Land plants, which evolved in a higher UV-B climate than at this moment, have developed adaptations to UV-B radiation (Rozema *et al.* 2002; Rozema *et al.* 2005).

One of these adaptations is the accumulation of UV-B absorbing pigments, e.g. flavonoids and other phenolics occurring in the leaves of many plants as a protective UV-B screen. This phenolic screen absorbs UV-B radiation and is transparent for photosynthetically active radiation (PAR) (Landry *et al.* 1995; Greenberg *et al.* 1997; chapter 2 of this thesis). In addition, flavonoids have a UV-B protective role by scavenging ROS, which are produced in enhanced amounts in response to UV-B (Landry *et al.* 1995; Markham *et al.* 1998b; Olsson *et al.* 1998). Furthermore, it is suggested that flavonoids dissipate the energy from the absorption of UV-B in a safe way (Smith & Markham, 1998).

In general, UV-B absorbing flavonoids are accumulating in high concentrations in the epidermal cells for optimal UV-B protection of mesophyll cells (Greenberg *et al.* 1997). The localisation of synthesis and accumulation of flavonoids may be in different sub cellular sites (Beerhues *et al.* 1988; Hutzler *et al.* 1998). Apart from the vacuole, some flavonoids are found to be covalently linked to plant cell walls or waxes (Strack *et al.* 1988; Schnitzler *et al.* 1996) and incidentally, cytoplasmic and nuclear flavonoids are observed in leaf tissues (chapter 2 of this thesis).

The localisation of the UV-B absorbing flavonoids is important for the effectiveness of the UV-B protection of photosynthetic tissues. A non-homogeneous distribution of flavonoids in the epidermis decreases the screening effectiveness in leaves due to the so-called "sieve effect". On the other hand, a non-homogeneous distribution of flavonoids in a transversal direction (unequal distribution over epidermis and mesophyll) could enhance the screening effectiveness (Vogelmann, 1994). The flavonoids of this UV-B screen are partly produced by a developmental program (constitutive flavonoids) and are partly synthesised in response to UV-B (UV-B induced flavonoids) (Winkel-Shirley, 2001).

The important UV-B protective role of constitutive and UV-B induced flavonoids is observed in leaves of many plants. In *V. faba* leaves, specific flavonoids like quercetin glycosides are induced in response to UV-B and contribute strongly to the UV-B induced attenuance. Moreover, kaempferol flavonoids, which are not UV-B induced, are important for the constitutive UV screen in the leaves (chapter 4 of this thesis). Kaempferol and quercetin have similar UV-B absorbing properties, but quercetin has one additional hydroxyl group in the B-ring, and therefore shows better reactive oxygen species (ROS)

scavenging and energy dissipation properties (Yamasaki *et al.* 1997; Smith & Markham, 1998). Although it is known which flavonoids are important for UV-B protection in leaves of *V. faba*, their localisation in response to UV-B irradiation is still unexplored.

We investigated the localisation of both kaempferol and quercetin flavonoids in *V. faba* leaf tissues in response to UV-B radiation because both flavonoids have similar UV-B absorbing properties and contribute to the constitutive and induced UV-B screen (Greenberg *et al.* 1997; chapter 2 of this thesis). The localisation was studied both in a qualitative way through visualisation by fluorescence microscopy and in a quantitative way by isolation of epidermis and mesophyll layers in which the flavonoids were measured. Since the existing methods for isolation of epidermal layers were unsatisfactory, more accurate methods have been developed.

Materials and methods

Growth conditions and UV treatments

Two cultivars of *V. faba* L. (cv. Minica and Pistache) were used for the experiments. Cv. Minica showed no reduced growth in response to UV-B whereas cv. Pistache was reduced in growth rate (chapter 1 of this thesis). However, the localisation of flavonoids appeared similar for both cultivars. Seeds were sown as described in chapter 3. Ten to fourteen days after sowing, just before the first leaves unfolded, the UV-B treatment was started in a greenhouse. The PAR flux was maintained to at least 250 μmol $m^{-2}s^{-1}$ (Philips HPI-T 400W) at canopy height. Greenhouse conditions and the manner of establishing the UV-B level were as follows: (1) "UV-B" treated plants were exposed to a UV-B biologically effective dose of 12 kJ m^{-2} day^{-1} at canopy height, weighted with the generalised plant action spectrum (GPAS; Caldwell, 1971). (2) The "UV-A" treated plants were irradiated with the same lamps wrapped in Mylar foil (thickness 0.13 mm), to filter out radiation below 313 nm but transmitting UV-A. (3) In the control treatment ("PAR"), no UV lamps were lit above the plot, so that the plants only received PAR and no additional UV-A and UV-B radiation. Plants were rotated within the treatments twice a week to avoid site effects. Furthermore, treatment blocks were rotated once a week within the greenhouse.

Experimental design

In the *in vitro* experiments the flavonoids were analysed quantitatively in the leaf tissue layers by HPLC to corroborate the qualitative microscopic data. After 20 days of

treatment, leaf number 4 (mature leaf) was harvested to isolate the tissue layers with the scrape method (see isolation tissue layers). After 23 days of treatment, leaf number 5 was harvested (mature leaf) to isolate the tissue layers with the strip method. Leaf number 4 and 5 were both in the same developmental stage at the moment of harvest, were irradiated for the same duration and originated from the same batch of *V. faba* plants (cv. Minica). Per treatment (UV-B, UV-A and PAR), four leaf replications were taken. Leaf tissue layers were isolated in two ways with the "scrape" method (one adaxial or abaxial epidermal layer plus mesophyll removed, one epidermal layer remained) and "strip" method (one adaxial or abaxial epidermal layer stripped, mesophyll and other epidermal layer remained). The flavonoid concentrations in the remaining epidermis and mesophyll layers were analysed by HPLC.

In the *in vivo* experiments flavonoids were examined qualitatively in leaf number 5 (mature developmental stage) of *V. faba* plants (cv. Minica and Pistache) that were UV-B irradiated during 27 days. Sections were examined with the fluorescence microscope in two ways with the colouring agents Naturstoffreagenz A (diphenylboric acid 2-aminoethyl ester) and ammonia.

Isolation, extraction and HPLC analysis of flavonoid aglycons in leaf tissue layers

To analyse the flavonoids in epidermis and mesophyll tissues separately, the layers were isolated in two ways i.e. with the "scrape" and "strip" method. The isolation of the epidermis for analysis by carefully peeling off the epidermis was considered. However, as Weissenböck *et al.* (1984) concluded, this method was not satisfactory since only 50% of the cells remained intact. Therefore, new scrape and strip methods were developed.

For the scrape method the adaxial epidermis, abaxial epidermis and whole leaf were sampled. To obtain these samples, the abaxial epidermis was removed (peeled off) followed by scraping away carefully the mesophyll cells with a blunt knife so that the adaxial epidermal layer remained for analysis. The abaxial epidermis was obtained with a similar procedure. The remaining adaxial or abaxial epidermis was rinsed with an isotonic 0.5 M sorbitol solution. With the results of adaxial and abaxial epidermis and the whole leaf, the flavonoid content of the mesophyll was calculated. In contrast to this scrape method, with the method of Weissenböck *et al.* (1984), the adaxial and abaxial epidermis were obtained directly by peeling off carefully these layers from the mesophyll.

For the strip method the whole leaf, the whole leaf minus abaxial epidermis and the whole leaf minus adaxial epidermis were sampled. To obtain these samples, the adaxial or abaxial epidermis was stripped off. The remaining epidermis plus mesophyll were

rinsed in the 0.5 M sorbitol solution. Using the flavonoid concentrations in the remaining tissues, the flavonoid contents of epidermis and mesophyll were calculated.

The area of the epidermal (plus mesophyll) tissue was measured with a LI-3100 area meter (LI-COR inc.). The areas of the samples were between 1.5 and 5.0 cm^2. A sample of the whole leaf (2.5 cm^2) was also obtained by taking 5 discs of 0.5 cm^2 each. The tissue samples were frozen in liquid nitrogen and freeze dried for 48 hrs. After drying, the samples were weighted and flavonoids were extracted and analysed with HPLC.

Flavonoids in leaf discs were extracted twice in 2.5 mL 90 % methanol and twice in 2.5 mL 10 % methanol by shaking (60 rpm) alternating for 9 and 15 h in the dark at room temperature (20 ^{o}C). The four extracts were pooled. During the first extraction, a fixed amount of flavon was added as an internal standard. Extracts were hydrolysed by adding 0.4 mL concentrated hydrochloric acid (36 w/w %) to 4 mL of a centrifuged extract. Extracts with added acid were boiled in closed Pyrex tubes for 1 h at 100 ºC. After hydrolysis, methanol was evaporated. Demineralised water (5 mL) was added to the samples together with 3 mL ethylacetate. Tubes were shaken vigorously and the ethylacetate fraction containing the hydrolysed flavonoids was saved. Flavonoids in hydrolysates were extracted a second time using 1.5 mL ethylacetate. The pooled ethylacetate fractions were dried under reduced pressure in a speed-vac. Dried samples were stored at 4 ºC until HPLC analysis.

Before analysis, samples were dissolved in 200 μL of methanol with 0.1 % trifluoracetic acid (TFA) and filtered over a 0.2 μm nylon centrifuge filter (Costar Spin-X). The samples were run on a gradient of acetonitril (A) and water (B) with 1 % TFA at 60 ºC at a flow rate of 0.6 mL min^{-1}. The stepwise linear gradient started isocratically for 1 min with 8 % (A), then 8 to 16 % (A) for 29 min, 16% to 34 % (A) for 7.75 min, 34 % to 81 % (A) for 15.25 min, 81 % to 95% (A) for 2 min, isocratically 95 % (A) for 10 min, back to 8% (A) in 2 min and finally kept on 8% (A) for 13 min. The HPLC system was a Waters 2690 Alliance system equipped with two C-18 columns (3.9*150 mm) (Waters Nova Pak) and a C-18 guard column (Waters), with a Photo Diode Array detector (Waters 996).

For identification and quantification of the flavonoid aglycons in the extracts, calibration curves were made with commercial standards of flavon (Fluka), kaempferol (Roth) and quercetin (Sigma). Identification of kaempferol and quercetin aglycons was done by comparing retention times of the samples with the purified aglycons. In addition, the UV spectra of the identified peaks were compared to the UV spectra of the purified aglycons. Flavonoid peaks were integrated by absorption at 254 nm for quantification.

For the scrape isolation method, it was checked with a microscope if the epidermal cells remained intact so that the flavonoids could not leak away. Viable cells were able to take up neutral red in the vacuole, which coloured the content of the vacuole red. For this test, neutral red stock solution (5 g/L) was diluted 50 times with a sodium carbonate solution (2.5 g/L). Adaxial and abaxial epidermal layers, isolated with the scrape method, were incubated for 5 min before being checked with the microscope. More than 90 % of the cells of the samples absorbed the neutral red in the vacuole and thus demonstrated to have an intact vacuole with a functioning membrane after isolation. The samples used for these viability tests were not used for the flavonoid analysis.

Examination of the flavonoids in leaf tissue by fluorescence microscopic techniques

Cross sections of fresh *V. faba* leaves were prepared by cutting the leaves held between two pieces of polypropylene foam, with a razor blade and preserved in a sample buffer (0.1 M potassium phosphate; pH=6.8). For visualisation of the flavonoids, the sections were incubated for 5 min in the fluorescence inducing Naturstoffreagenz A (NA), or in the fluorescence shift reagent ammonia (0.5 %). Both compounds were staining reagents for flavonoids by fluorescence microscopy (Hutzler *et al.* 1998). Fluorescence tests with kaempferol and quercetin standards with an LS-50-B spectrofluorimeter (Perkin Elmer, Beaconsfield, UK) showed that in ammonia reagent, fluorescence of quercetin decreased strongly within a few minutes whereas fluorescence of kaempferol remains for at least 30 minutes. In NA reagent, fluorescence of both compounds remained for at least 30 min. The NA solution consisted of potassium phosphate buffer (0.1 M, pH 6.8), NA (1µg/ml), Gramicidin-D (0.7 µg/ml) and ammonium chloride (5 mM) to make the pH of the vacuole more basic so that the NA could react with the flavonoids in the vacuole (Chardonnens *et al.* 1999).

When kaempferol and quercetin flavonoids were not located at the same site in the cell and tissue, both flavonoids could be distinguished in the sections dyed with NA (Sheahan *et al.* 1998). After treatment with ammonia (0.5 % in phosphate buffer, pH=6.8), the resulting quercetin-derivate was short-lived and therefore, fluorescence of quercetin decreased strongly within a few minutes. Thus after dying with ammonia, mainly the fluorescence of kaempferol was visible as a light green colour (maximum at 555 nm) (own observation).

The fluorescence microscope (Leica DM RHC) was equipped with Xe 75 and Hg 50 lamps and PL Fluotar 10, 20 and 40 objectives were used. Two filter blocks were used: For the ammonia dyed cross sections with UV excitation, a BP (band pass) filter of 340-380 nm was used in combination with a LP (long pass) 430 filter. For the blue excitation

of the Naturstoffreagenz A dyed sections, a BP filter of 450-490 nm was used in combination with an LP 515 filter. Photographic documentation was performed with a digital camera (Spot junior 1.5, Diagnostic Instruments USA.) and software (Spot 32, version 2.1).

Statistics

Data were tested statistically using SPSS software (SPSS Inc. version 8.0). Normality was tested with Shapiro-Wilk and homogeneity of variance was tested with Levene's test. Kaempferol and quercetin content per leaf area were square root transformed, to obtain homogeneity of variance for the ANOVA. Per epidermal isolation method, a one way ANOVA was performed with treatment as independent factor to analyse treatment differences between the leaf layers. UV-A and UV-B effects were analysed with paired comparisons of the "PAR" (without additional UV light), "UV-A" (additional UV-A light) and "UV-B" treatments with a Tukey HSD post-hoc test.

Comparison of both epidermal isolation methods was analysed non-parametrically with the Wilcoxon signed rank test. Also differences between the leaf layers (adaxial and abaxial epidermis and mesophyll) were analysed non-parametrically per treatment with the Wilcoxon signed rank test (Sokal & Rohlf 1995).

Figure 2 (next page): Fluorescence microscopy images of ammonia induced fluorescence in cross sections of *V. faba* leaves (cv. Minica and cv. Pistache) exposed to no additional UV-A or UV-B (PAR) or to additional UV-A +UV-B (UV-B). Horizontal thick white bar represents 100 μm. Excitation at 340-380 nm. LP filter 430. Localisation of kaempferol is indicated in figures with "K". "ab epid": abaxial epidermis, "ad epid": adaxial epidermis, "pp": palisade parenchyma, "sp": spongy parenchyma, "gc": guard cell.

A: cv. Pistache, UV-B treatment **B**: cv. Pistache, PAR treatment

C: cv. Minica, UV-B treatment **D**: cv. Minica, UV-B treatment

Figure 3 (next page). Fluorescence microscopy images of ammonia (A) (Excitation at 340-380 nm, LP filter 430) and Naturstoffreagenz A (B) (Excitation at 450-490 nm, LP filter 515) induced fluorescence of adaxial epidermis of *V. faba* leaves (cv. Minica) exposed to additional UV-A +UV-B (A) or without additional UV radiation (B). Horizontal thick white bar represents 100 μm. Localisation of kaempferol is indicated in figures with "K". "gc": guard cell, "tr": trichome

A: UV-B treatment **B**: PAR treatment

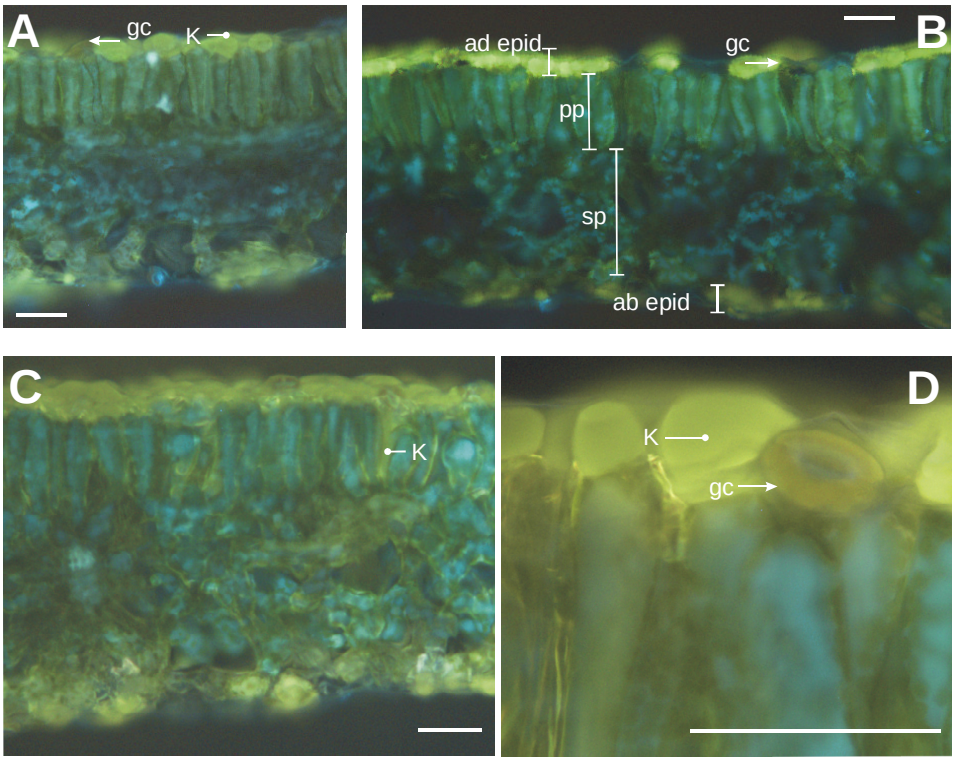

Figure 2 (caption on previous page)

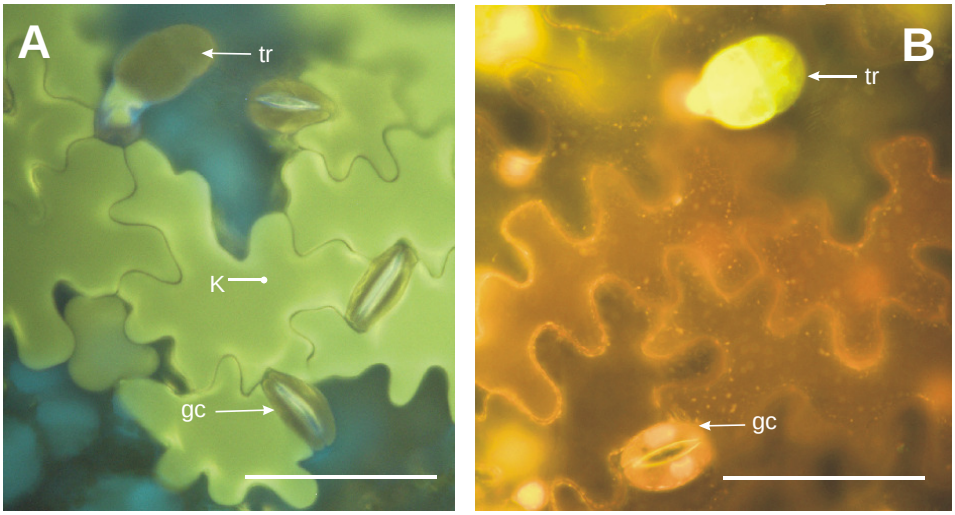

Figure 3 (caption on previous page)

Results

Quantitative distribution of flavonoids over the leaf

Kaempferol flavonoids concentrations, determined quantitatively by HPLC, did not differ when the epidermal layers were isolated with the scrape or with the strip method (Figure 1A and C). Also quercetin concentrations were similar for both stripping methods, except for the quercetin concentration in the abaxial epidermis, which was higher with the strip method than with the scrape method in all UV treatments ($p=0.028$) (Figure 1B and D).

The kaempferol content of the whole leaf did not change significantly in response to UV-B and varied around 0.035-0.05 μmol/cm^2 (Figure 1A and C). All treatments showed the same distribution of kaempferol over the three tissue layers (adaxial, abaxial epidermis and mesophyll), whereby kaempferol content was significantly higher in the adaxial epidermis than in the abaxial epidermis ($p=0.012$ and $p=0.022$ for scrape and strip method, respectively).

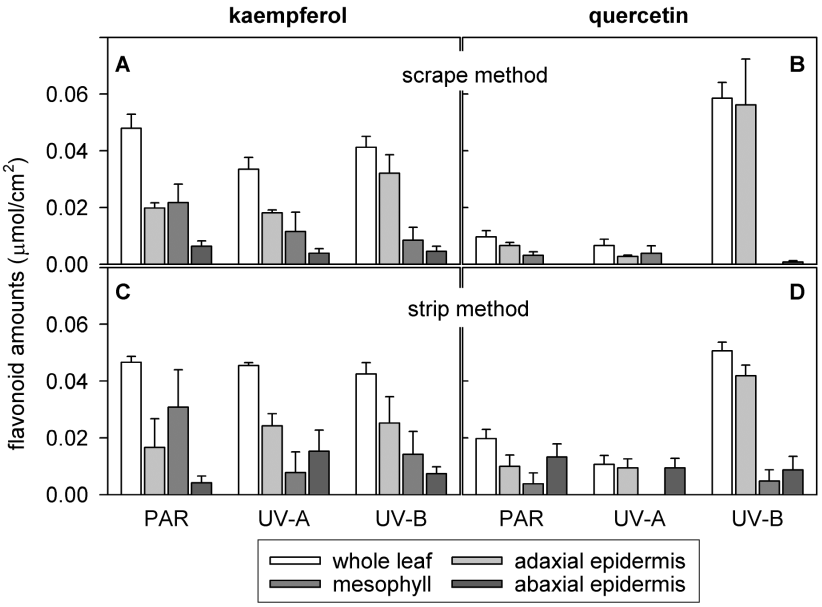

Figure 1. Kaempferol (A and C) and quercetin (B and D) content (μmol cm^{-2}) in whole leaf, adaxial epidermis, mesophyll and abaxial epidermis of *V. faba* exposed to no additional UV-A or UV-B (PAR), to additional UV-A (UV-A), or to additional UV-A +UV-B (UV-B). Epidermal isolation method according to scrape method (A and B) and strip method (C and D). Data are means ± SE (n=4).

In contrast, the content of quercetin in the whole leaf increased strongly in response to UV-B (Figure 1B and D) ($p<0.001$ for both stripping methods). This increment was due to the increase of quercetin in the adaxial epidermis in response to UV-B ($p=0.017$ for the scrape method, $p< 0.001$ for the strip method). The quercetin content of the whole leaf and the adaxial epidermis increased from 0.01-0.02 to 0.05-0.06 $\mu mol/cm^2$. The quercetin level in the abaxial epidermis and mesophyll was not significantly enhanced in response to UV-B. In response to UV-A, the quercetin concentrations remained low in all tissue layers (compare UV-A and PAR treatments in Figure 1).

In all treatments the quercetin content in the mesophyll was lower than the kaempferol content ($p=0.028$ and $p=0.015$ for the scrape and strip method, respectively). Moreover, in the *minus* UV-B treatments (thus PAR and UV-A treatment) kaempferol concentrations of both epidermal layers were also higher than quercetin concentrations. In contrast, quercetin concentrations of the whole leaf and the adaxial epidermis were higher than kaempferol concentrations after UV-B irradiation (Figure 1).

Qualitative distribution of flavonoids over the leaf

Kaempferol localisation visualised with ammonia induced fluorescence.

The presence of kaempferol was demonstrated as a light green fluorescing colour in sections treated with ammonia (indicated with letter K in Figures 2A, 2C, 2D, 3A). The fluorescence of quercetin flavonoids was quenched after some minutes and was therefore not visible in the ammonia treated sections (Figures 2A to 2D and 3A). No differences were observed between *plus* and *minus* UV-B treated sections dyed with ammonia (compare Figures 2A and 2B) or between both cultivars in ammonia dyed sections (compare Figures 2A and 2C).

The epidermal layers contained higher concentrations of kaempferol than the mesophyll (Figure 2A to 2C). The accumulation in the adaxial epidermis was higher than in the abaxial epidermis (Figure 2B). The light green fluorescence (indicating kaempferol) was brighter in the palisade parenchyma than in the spongy parenchyma (Figures 2A and 2B).

The adaxial part of a UV-B treated leaf is shown in Figure 2D. Epidermal cells were filled abundantly with kaempferol whereas guard cells (in figures indicated as gc) were less fluorescing and thus contained less kaempferol. The cytoplasm of the palisade parenchyma contained small amounts of kaempferol (Figure 2C).

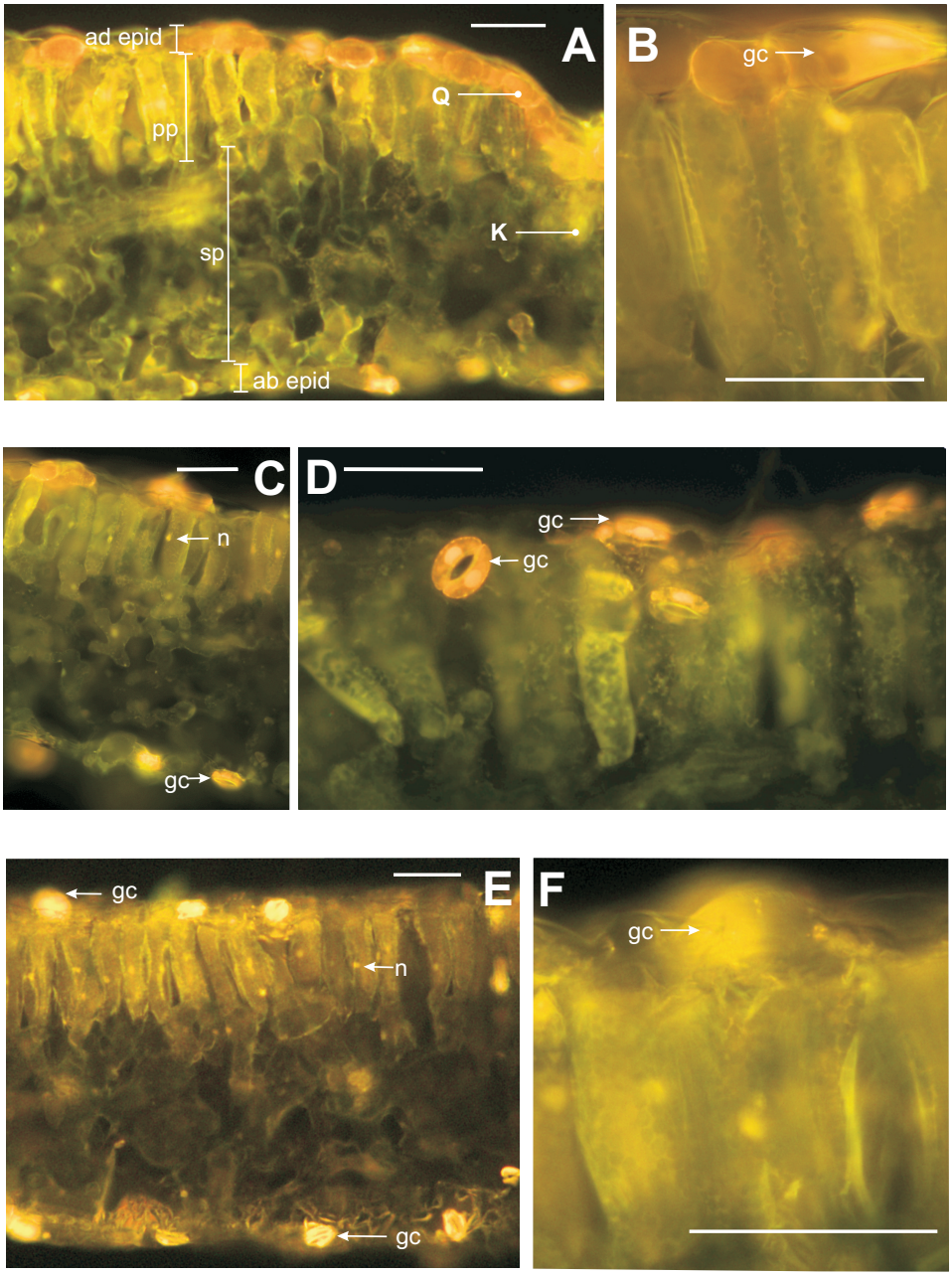

Figure 4 (caption on next page)

When viewing the adaxial epidermis from above, the epidermal cells filled with kaempferol appeared like jigsaw pieces (Figure 3A). Again, the guard cells were less fluorescent, indicating that they contained less kaempferol than the surrounding epidermal cells. In this image a trichome-like bulge was visible. Because some epidermal cells were disrupted during the section preparation, the underlying palisade parenchyma residues (blue) were slightly visible. Kaempferol flavonoids in the epidermal cells were mainly located in the vacuole (Figures 2D, 3A).

Kaempferol and quercetin localisation visualised with Naturstoffreagenz A induced fluorescence

Examination of leaf sections incubated with Naturstoffreagenz A (NA) showed fluorescence of quercetin and kaempferol in the leaves after blue excitation (450-490 nm). Similar to the ammonia dyed sections, no differences in localisation of quercetin and kaempferol flavonoids between sections of equally treated plants of cv. Pistache and cv. Minica were observed (compare UV-B in Figures 4A and 4C with PAR in Figures 4D and 4E). Therefore, no distinction between images of both cultivars was made to illustrate different treatments. In addition, no apparent differences between PAR and UV-A treated sections were observed (compare Figures 4D to 4F). Therefore, a representative selection of the two treatments, PAR and UV-A was made and both treatments are further indicated as "*minus* UV-B treatment".

Representative cross sections of UV-B treated leaves dyed with NA are shown in Figures 4A and 4C. The yellow colour reflects the fluorescence of kaempferol flavonoids (indicated with K in Figure 4A). The orange colour is originating from fluorescence of quercetin flavonoids (indicated with Q in Figure 4A) (Sheahan *et al.* 1998), which mainly accumulated in the adaxial epidermis and to a lower extent in the abaxial epidermis. When a compartment contained quercetin and kaempferol, the colour of the fluorescing light was a mixture between yellow and orange. Therefore yellow compartments mainly

Figure 4 (previous page). Fluorescence microscopy images of Naturstoff reagenz A induced fluorescence in cross sections of *V. faba* leaves (cv. Minica and cv. Pistache) exposed to no additional UV-A or UV-B (PAR), to additional UV-A (UV-A), or to additional UV-A +UV-B (UV-B). Horizontal thick white bar represents 100 μm. Excitation at 450-490 nm. LP filter 515. Localisation of kaempferol and quercetin are indicated in figures with "K" and "Q" respectively. "ab epid": abaxial epidermis, "ad epid": adaxial epidermis, "pp": palisade parenchyma, "sp": spongy parenchyma, "gc": guard cell, "n": nucleus.

A: cv. Minica, UV-B **B**: cv. Pistache, UV-B **C**: cv. Pistache, UV-B
D: cv. Minica, PAR **E**: cv. Pistache, PAR **F**: cv. Pistache, UV-A

contained kaempferol, but it is possible that also small amounts of quercetin were present in this compartment. Similar limitations occurred for the orange compartments. It was not possible to compare the fluorescence intensities between different pictures quantitatively since the brightness was optimised per image during image capture.

In both treatments the brightness of the fluorescence was higher in the guard cells than in the surrounding epidermal cells (Figures 3B, 4C to 4F). The guard cells of the adaxial side of UV-B treated leaves contained higher amounts of quercetin than the adjacent epidermal cells (Figures 3B, 4C) and in turn, the epidermis as a whole was more orange and thus contained more quercetin than the palisade parenchyma (Figures 4A to 4C). In the adaxial epidermis of UV-B treated leaves the orange fluorescence of quercetin was predominant (Figures 4A to 4C). This was less evident in *minus* UV treated leaves (Figures 4E, 4F).

In all treatments the adaxial epidermis had a more intense fluorescence than the abaxial epidermis (Figures 4A, 4C and 4E). Furthermore, the mesophyll cells of *plus* and *minus* UV-B treatments mainly contained kaempferol (yellow fluorescence). The accumulation of the flavonoids within the mesophyll appeared to be higher in the palisade parenchyma than in the spongy parenchyma (Figures 4A, 4C and 4E).

In all treatments and cultivars, flavonoids occurred in higher concentrations around the nucleus of mesophyll cells and guard cells than in the other parts of these cells (Figures 3B, 4C, 4E, 4F*)*. Furthermore, on a subcellular level, the flavonoids were located in higher concentrations within in the vacuole of epidermal cells (Figures 3B, 4A).

Trichomes were observed on the adaxial epidermis (Figure 3B). The colour of the trichome (*minus*-UV-B) differed from the epidermis and guard cells, indicating that the trichome did not contain quercetin or kaempferol but another compound. This figure was comparable to Figure 3A (dyed with ammonia) in which also a similar trichome structure and guard cells were observed.

Discussion

Localisation of flavonoids coupled to their function in UV-B protection

V. faba leaves contained both UV-B induced quercetin flavonoids and constitutive quercetin and kaempferol flavonoids (Figure 1). We consider flavonoids synthesised in the *minus* UV-B treatments to be constitutive because other environmental factors such as high PAR levels or infections (Tattini *et al.* 2000; chapter 3 of this thesis) could not play a role in our experimental set-up. Both constitutive and induced flavonoids form a UV-B absorbing screen to protect the leaves against harmful UV-B radiation. This UV-B

screen is transparent for PAR, which can reach the mesophyll photosynthetic tissues nearly without attenuation. In *V. faba* the constitutive flavonoids in the whole leaf provide a good UV-B filter, which absorbs 99.9 % of the UV-B radiation (chapter 4 of this thesis). Beside factors as UV-B absorbing properties and concentration of the phenolics, the localisation of UV-B induced and constitutive phenolics is important for the screening effectiveness (Vogelmann, 1994; Bornman, 1999).

UV-B induced quercetin accumulation was restricted to the adaxial epidermal layer in *V. faba* (Figures 1A to 1D, compare Figures 4A, 4B with 4E, 4F). Also in many other plant species flavonoids with an additional hydroxyl group in the B ring like quercetin are induced by UV-B radiation (Schnitzler *et al.* 1996; Olsson *et al.* 1999). Kaempferol flavonoids (which are never UV-B induced in *V. faba* leaves) and small amounts of constitutive quercetin flavonoids were present in all leaf layers although the adaxial epidermal layer and the palisade parenchyma contained higher concentrations than the abaxial epidermal layer and spongy parenchyma (Figures 1A to 1D, 2B, 4E). Thus in general, in both *plus* and *minus* UV-B treated *V. faba* leaves, a flavonoid concentration gradient across the leaf from the adaxial to abaxial side was observed (Figures 4A, 4C and 4E).

This non-homogeneous distribution in the leaf, with higher concentrations of flavonoids in the upper leaf layer, contributes to an effective screen (Vogelmann 1994; Markstädter *et al.* 2001). The quercetin and kaempferol flavonoids localized in vacuoles of the adaxial epidermis (Figures 3A, 3B, 4A, 4B, 4E) filter out the UV-B light before the damaging radiation can reach the mesophyll. However, the non-uniform epidermal UV screen with higher concentrations of quercetin in guard cells and anticlinal epidermal cell walls on one hand and the aperture of the stomatal pores on the other, reduces the UV-B screening effectiveness (Vogelmann 1994; Bornman 1999; Figures 3A, 3B and 4A, 4E). In spite of these gaps, the distribution seems to be homogeneously enough to avoid severe UV-B damage as was observed in tolerant *Arabidopsis* mutants (Bieza & Lois, 2001). Other phenolics such as hydroxycinnamic acids are constitutive in most plant species (chapter 2 of this thesis; Bornman 1999) and were mainly observed in the mesophyll where a blue fluorescence could be noted (Figures 2B, 2C; Schnitzler *et al.* 1996; Kolb *et al.* 2001). These hydroxycinnamic acids have UV-B absorbing properties and contribute to the constitutive UV-B screen (Landry *et al.* 1995; Bornman 1999).

The UV-B induced flavonoids, localized in the adaxial epidermis, are frequently mentioned as an important UV-B screen (chapter 2 of this thesis; Bornman 1999; Markstädter *et al.* 2001). In contrast, the role of the constitutive phenolics (kaempferol and hydroxycinnamic acids) occurring in the mesophyll is often neglected or underestimated as UV-B protection (Olsson *et al.* 1999; Kolb *et al.* 2001; Markstädter *et*

al. 2001). Indeed, these constitutive mesophyll flavonoids are probably less effective as general UV-B screen for the photosynthetic tissues. However the subcellular accumulation of these mesophyll flavonoids around nucleus and chloroplasts provides microscreens to protect target organelles from UV-B radiation (Figures 4B to 4F; Sheahan *et al.* 1998). It is a second trap on these specific sites for UV-B radiation that passed through the epidermal screen. Association of flavonols sulphate esters with the nucleus has also been demonstrated in *Flaveria chloraefolia* and *Arabidopsis thaliana* (Grandmaison & Ibrahim 1996; Sheahan *et al.* 1998). Thus in addition to heterogeneous localisation within the tissues, the flavonoids in *V. faba* leaves also seemed to be partly subcellularly sequestered (Figure 4C, 4E; Beerhues *et al.* 1988). Although different colours in fluorescing spots in the cells were observed, kaempferol and quercetin flavonoids are supposed to co-exist at these subcellular sites because there is no evidence for such differential subcellular sequestration (Sheahan *et al.* 1998). It appears that quercetin dominated in the cytoplasm of the mesophyll and guard cells whereas kaempferol was highly fluorescing in the vacuole of epidermal cells (Figures 2D and 3A).

Quercetin and kaempferol flavonoids have similar UV-B absorbing properties and are therefore equally suitable as UV-B screening agent. However, it is remarkable that in response to UV-B, only quercetin flavonoids are induced whereas in the *minus* UV-B treated leaves, kaempferol is the dominant flavonoid (Figure 1). The flavonoids have other characteristics that could be helpful in UV-B protection like dissipation of energy and scavenging ROS. In this respect, quercetin has more effective properties than kaempferol flavonoids because quercetin flavonoids have a higher reactive oxygen species (ROS) scavenging capacity and energy dissipation effectiveness than kaempferol flavonoids (Yamasaki *et al.* 1997; Smith & Markham *et al.* 1998b). More ROS are induced in response to UV-B radiation, so it is essential to scavenge these ROS in the chloroplasts to prevent damage to photosynthesis, and in the nucleus to prevent damage to DNA (Greenberg *et al.* 1997). Since these ROS are produced in the cytosol, cytoplasmic quercetin flavonoids are more suitable for this scavenging function than vacuolar kaempferol flavonoids (Yamasaki *et al.* 1997).

Specialised epidermal structures

Guard cells contained mainly quercetin flavonoids and kaempferol could be ruled out since the guard cells did not fluoresce brightly in the ammonia dyed sections (Figures 2A, 2B, 2D, 3A). These cells contained more quercetin and less kaempferol than the surrounding epidermal cells (Figure 2D). This quercetin was at least partly constitutive (Figures 1, 3B and 4D). Results of other researchers also indicated higher levels of

flavonoids in guard cells of several herbaceous species (Beerhues *et al.* 1988; Tattini *et al.* 2000). There was no significant increase in the number of stomata in plant species in the family of Fabaceae like *V. faba* and pea with enhanced UV-B (Visser *et al.* 1997; Nogués *et al.* 1999). Therefore an increase in the number of stomata cannot explain the increase in adaxial quercetin content.

The concentrated localisation of quercetin in guard cells did not seem to be functional in general screening of UV-B since it resulted in an inefficient heterogeneous screen for the whole leaf. However, microscreens (i.e. regions with higher fluorescence) around the nucleus of guard cells (Figure 3B) indicated a role in protecting the DNA in the nucleus against harmful UV-B radiation (Grandmaison & Ibrahim 1996). Other specific functions of high flavonoid levels in the guard cells remained unclear and complex. It was suggested that a flavonoid was the chromophore of a UV-B receptor (Eisinger *et al.* 2003), which was involved in stomata aperture and differentiation (Liu-Gitz *et al.* 2000).

Some trichomes were noticed on the epidermis. In the ammonia dyed section the trichomes and guard cells were coloured equally dark (Figure 3A). However, in the NA dyed section (Figure 3B), the trichome had a different (not orange) colour than the guard cells so that a considerable quercetin and kaempferol content could be ruled out. The basal cells of the trichome had the same fluorescing characteristics as the guard cells, thus probably contained quercetin. In some plant species with high trichome densities such as *Olea europaea*, *Quercus ilex* and *Phillyrea latifolia* these extensions could contribute to the UV-B screen (Karabourniotis *et al.* 1998; Tattini *et al.* 2000). Although the phenolics in trichomes had UV-B absorbing properties (Bieza & Lois 2001; Bornman 1999), the density of trichomes in *V. faba* were regarded too low (Figure 3) to contribute considerably to a UV screen.

It can be concluded that the localisation of flavonoids is the result of induction by UV-B of quercetin in the adaxial epidermis and constitutive flavonoid synthesis, which is part of a tissue specific differentiation program. These constitutive flavonoids are kaempferol in both epidermis and mesophyll and quercetin in mesophyll and abaxial epidermis. In the leaf, there is a flavonoid concentration gradient with highest concentrations in adaxial epidermis, lower concentrations in palisade parenchyma and lowest concentrations in spongy parenchyma. This distribution of flavonoids over the leaves contributes to an optimal filtering of UV-B radiation, which is supposed to be one of the most important functions of flavonoids in the leaves. Microscreens of flavonoids around nucleus and chloroplasts of palisade parenchyma and guard cells provide additional UV-B protection. The functions of the very high levels of quercetin especially in the guard cells are fascinating but still not elucidated.

Acknowledgements

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The canopy structure of *Vicia faba* is denser in response to UV-B.

Chapter 6

General discussion

General discussion

In this thesis, the effects of, and adaptations to enhanced UV-B radiation were studied in a greenhouse at three levels: the whole-plant, the leaf tissues and the cells. Thereto, two *V. faba* cultivars (Minica and Pistache), differing in UV-B growth response were investigated. These two cultivars were selected in a "screening experiment", in which the growth responses to UV-B of 5 cultivars of *V. faba* were measured (Chapter 1). First, the use of greenhouse experiments for studying plant adaptations to UV-B radiation is discussed. Next, the observed whole-plant responses of the two *V. faba* cultivars and the leaf tissue responses are considered. With respect to the leaf tissue responses the focus is on attenuance of UV-B radiation in the leaves and its underlying mechanisms: leaf morphology, leaf chemistry and the localisation of UV-B absorbing compounds in the leaves. Based on observations of specific intracellular flavonoid localisation, some remarks are made on cellular UV screening by what I called "microscreens". Finally, the main conclusions of this thesis are presented. The discussion is organised around Figure 1, in which the attenuation of UV-B radiation is treated at three levels: the whole-plant level, the tissue level and the cellular level.

Greenhouse studies with low PAR levels on *V. faba*: a useful model system to investigate UV-B responses?

Negative UV-B effects on terrestrial plants can be overestimated in growth chamber and greenhouse experiments due to lower PAR levels in these indoor environments in comparison with the field situation (Runeckles & Krupa 1994; Day & Neale 2002; Krizek 2004). PAR levels as occurring in the field can ameliorate the negative UV-B effects because PAR has a positive effect on the DNA repair enzyme photolyase and it enhances the production of polyamines, which protect the membranes. In addition, higher PAR levels can induce morphological alterations like more branching, shorter plants and thicker leaves, which are favourable traits in a high UV-B environment (Chapter 3; Krizek 2004).

Despite the more realistic light circumstances in field experiments, greenhouse experiments were used for the investigation of adaptation mechanisms to UV-B in plants described in this thesis. The advantages of greenhouse studies are numerous because experimental conditions can be better controlled (Krupa & Kickert 1989). This is very important for the experimental set up because plant architecture, leaf morphology and phenolics content, which all play an important role in UV-B protection, are also influenced

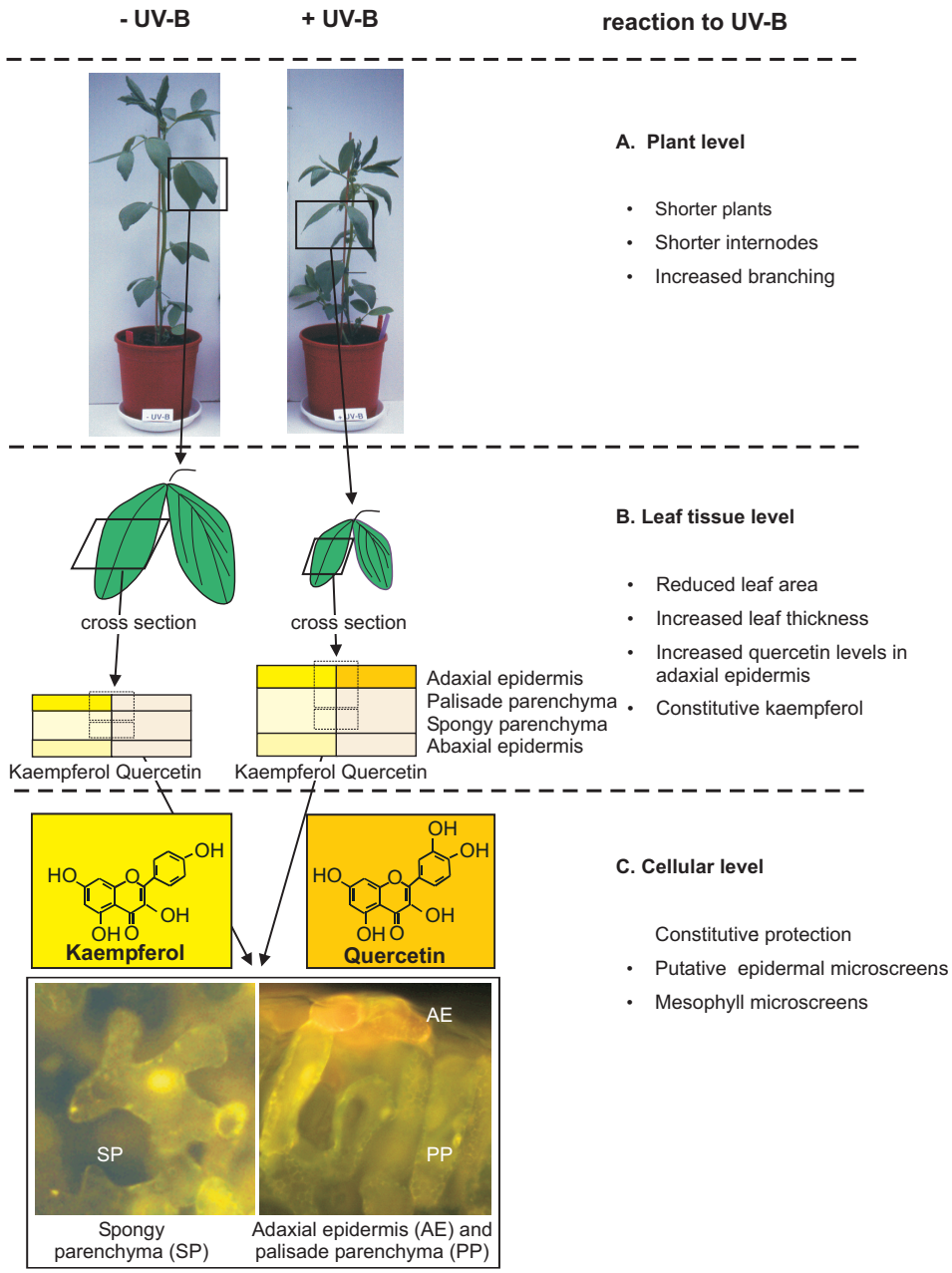

Figure 1. Schematic overview of the multilevel UV attenuance in *V. faba* leaves. Attenuance is determined by several factors on plant level (part A), leaf tissue level (part B) and cellular level (part C). The reaction to UV-B radiation (in comparison with *minus* UV-B radiation) is indicated. Part B: The relative distribution of quercetin (right side of the "cross section") and kaempferol (left side) in the leaves is visualised with the shade of the colours. The dotted boxes refer to details, visualised in figure C: the upper epidermal part of the leaf and spongy parenchyma.

by other environmental factors like herbivores, plant pathogens, water availability and temperature (Chapter 2; Runeckles & Krupa 1994; Caldwell *et al.* 2003).

In experiments performed in September and October, where PAR levels in the greenhouse were enhanced with lamps to near-ambient levels (Chapter 3), a positive growth response was observed in response to UV-B. The UV-B and PAR responses had similar photomorphogenic characteristics: branching and leaf thickness were increased, and plant height (internode distance) and leaf area were reduced at higher PAR or UV-B levels. Also UV-B radiation and PAR enhanced the concentration of UV-B absorbing pigments in the leaves. The effect of UV-B and PAR was additive for pigments but not for morphological characteristics (Chapter 3).

These comparable UV-B and PAR photomorphogenic responses suggest that a photoreceptor-mediated response is involved, which is at least partly similar for UV-B and PAR (Kakani *et al.* 2003; Krizek 2004; Chapter 3). It is not likely that there is only one general receptor but that several photoreceptors such as phytochrome, blue/ UV-A and UV-B receptors are responsible for the final PAR and UV-B responses (Frohnmeyer & Staiger 2003; Stratmann 2003; Krizek 2004).

The lower PAR level in the greenhouse and the unnatural light quality of the greenhouse environment in comparison to field situations can have subtle effects on plant morphology and plant chemistry, indirectly affecting the UV-B sensitivity of the whole plant. However, the more controlled circumstances in the greenhouse made it easier to relate effects to the UV-B treatment and not to other environmental conditions.

Whole plant UV-B responses: Growth and morphology (Figure 1A).

Although high levels of UV-B radiation ($UV\text{-}B_{BE}$: 12 kJ m^{-2} d^{-1}) were used, growth was increased in cv. Minica due to increased branching and number of leaves (Chapter 1; Chapter 3). Concomitant with increased branching also the biomass of the adventitious shoots increased. The adventitious branches in cv. Minica also bear leaves, which contribute to photosynthesis. The UV-B induced growth of this first adventitious shoot compensated for the growth reduction of the main shoot in response to UV-B (Chapter 3, see figure 1A). Also, other terrestrial plant groups, previously assumed to be sensitive to UV-B (Rozema *et al.* 2002), e.g. flowering tundra plants and polar bryophytes appeared to be insensitive for the enhanced UV-B levels at this moment occurring on Earth (Boelen *et al.* 2006; Rozema *et al.* 2005).

The appearance of the near-ambient PAR and UV-B exposed plants resembled plants with a loss in apical dominance (more branching and increased leaf thickness) (Singh 1996; Jansen *et al.* 1998; Chapter 3). A lot of these symptoms may be related to a reduction of auxin levels. After exposure to enhanced UV-B radiation or enhanced PAR,

active IAA (indoleacetic acid) concentrations in the plant can be reduced by enhanced photo-oxidation or binding of quercetin flavonoids to IAA (Runeckles & Krupa 1994; Jansen *et al.* 1998; Taylor & Grotewold 2005).

This change in plant architecture and leaf morphology is probably an indirect effect because active auxin levels may be modified which in turn regulate the plant morphology. This more compact plant architecture is a first barrier to harmful UV-B radiation. Due to these changes, less UV-B radiation can penetrate the canopy and reach the leaf tissues where it may damage the photosynthetic mesophyll. Thus, this more compact plant architecture can be considered an adaptation through which UV-B radiation is attenuated at the level of individual plants (See figure 1A).

The plant growth experiment described in Chapter 3 illustrates how damaging and adaptation processes are interwoven. Biomass accumulation of the main shoot and leaf area were reduced. However, this reduction in biomass accumulation was compensated by altered plant architecture (branching) so that the net effect was an increase in growth. It demonstrates that ranking of sensitivity to enhanced UV-B radiation based on six morphological, chemical and growth parameters, such as explained in Chapter 1, is better than one based on total growth only. In fact, in spite of the positive growth response to UV-B, it is possible that the individual leaves of cv. Minica are sensitive to UV-B (e.g. reduced photosynthesis) and are less productive in response to UV-B. Adventitious stems may compensate for this lower productivity of the leaves of the main stem. Thus, besides studying whole plant responses also responses on individual leaves are necessary to get insight into adaptation mechanisms. Therefore, I further investigated the attenuation of UV-B light by individual leaves as an adaptation to enhanced UV-B.

Leaf tissue UV-B responses: Attenuance of UV-B radiation (Figure 1B)

A very important protection to UV-B damage is the attenuation of UV-B radiation by UV screens consisting of UV-B absorbing compounds (e.g. flavonoids) in the leaves (Jansen *et al.* 1998; Chapter 2). It forms a second barrier for UV-B radiation. This barrier is based on increased leaf thickness, reduced leaf area and flavonoids in the leaves (Figure 1B). As a result, UV-B damage in leaves is limited by absorption of UV-B radiation in these leaves so that it cannot reach targets such as photosynthetic tissues and DNA.

The aspects related to attenuance of UV-B on leaf tissue level are discussed in the sections below and are visualised in figure 1B. This figure shows that in response to UV-B, leaves become smaller and thicker (see cross section). Moreover, the relative distribution of quercetin (right side of the "cross section") and kaempferol (left side) in

the leaves is visualised with the darkness of the colours. Finally, the dotted boxes in the cross sections refer to details, visualised in figure 1C: the upper epidermal part of the leaf and spongy parenchyma.

In this thesis, realistic *in vivo* UV attenuance was measured of intact *V. faba* leaves of different ages. *In vivo* UV attenuance was defined as the negative logarithm of the transmittance of UV radiation through a leaf. The transparency of *V. faba* leaves for UV radiation was very low (Chapter 4). The method described in Chapter 4, differs from other research on UV transmittance in *V. faba* (Lautenschlager-Fleury 1955; Flint *et al.* 1985; Bilger *et al.* 1997; Barnes *et al.* 2000; Markstädter *et al.* 2001) in that the attenuance of polychromatic light through the intact *in vivo* leaf system was measured. In this way also reflection and non-homogeneous attenuation were taken into account. Moreover, *in vivo* UV attenuance was coupled to flavonoid content by extracting soluble compounds from the leaf discs. The *in vivo* attenuance of the decolorised (soluble compounds extracted) leaf discs was also measured to get insight in the contribution of leaf thickness, cell wall structures and non-soluble compounds to attenuance.

Even in non UV-B exposed leaves, the transmittance was at most 0.1 % of the UV radiation. Thus, the constitutive attenuance was very high. This attenuation even increased further in response to UV-B, to a UV transparency of 0.01 % (Chapter 4). As a result, penetration of the harmful UV-B radiation appeared to be effectively reduced in *V. faba* leaves, although leaves of other plant life forms may have higher attenuance levels (Day *et al.* 1992, 1993). The UV-B induced attenuance was built up during maturation of the leaves. The UV attenuance and thus the light intensity in and beneath the leaves is a function of several factors which are discussed below: 1) leaf morphology 2) leaf chemistry and 3) the localisation of the UV-B absorbing compounds such as flavonoids (See Figure 1, Chapters 2, 4 and 5).

Leaf morphology

Leaf thickness (expressed as Specific Leaf Weight: Dry weight/Unit leaf area) influences the pathlength of the light within the leaf, which can be seen as the morphological factor of UV attenuance (Figure 1B: thicker cross sections; Cen & Bornman 1993; Day *et al.* 1993; Visser *et al.* 1997; Chapter 3). Although, leaf thickness was increased in response to UV-B, correlation between SLW and *in vivo* attenuance was low (Chapter 4). Moreover, the *in vivo* attenuance of extracted leaf discs was equal for *plus* and *minus* UV-B treatments. Thus, all UV-B induced attenuance disappeared after extraction of the leaf discs (Chapter 4). Therefore, it is concluded that leaf thickness is not very important for UV-B induced attenuance in these two *V. faba* cultivars.

Still, leaf thickness is important as a constitutive UV screen because only 16 % of the UV radiation passed through the extracted leaf discs (attenuance of around 0.8) (Chapter 4). Herbaceous species with relatively thin leaves such as *V. faba* are therefore less adapted to UV-B radiation than other plant life forms with thicker leaves such as some shrubs and trees (Day *et al.* 1992). During maturation leaves become thicker which makes them less sensitive for UV-B radiation (Chapter 4).

Leaf chemistry

Flavonoids contributed largely to the *in vivo* UV attenuance although also leaf structure and hydroxy cinnamic acids and non-soluble compounds contribute to the *in vivo* attenuance (see Figure 1B; Chapter 2 and Chapter 4). Leaf flavonoid concentrations and *in vivo* UV attenuance were highly correlated in mature leaves (Chapter 4). Moreover, the UV-B induced attenuance (difference in attenuance of *plus* and *minus* UV-B treatment) for the calculated spectrum (based on flavonoid concentrations measured in the leaves) and observed spectra were equal. This implies that quercetin flavonoids were responsible for the UV-B induced attenuance (Chapter 4). The relative content of quercetin and kaempferol and distribution of the flavonoids in the different tissue layers are indicated as light (lower concentrations) or dark (higher concentrations) colours in figure 1B.

Kaempferol flavonoids are constitutive in the leaf whereas quercetin flavonoids are mainly induced by UV-B radiation. The total levels of flavonoids (quercetin plus kaempferol) in mature leaves were around 0.1 μmol cm^{-2} for *minus* UV-B and 0.2 μmol cm^{-2} for *plus* UV-B treated leaves (Chapter 4). However this total level may vary depending on differences in climate and light conditions in the greenhouse during the different experiments and depending on the age of the mature leaf (Chapters 2, 3, 4 and 5). In *V. faba* leaves, levels of constitutive kaempferol flavonoids, which are already synthesised in young leaves, decreased with maturation due to cell and leaf elongation whereas UV-B induced quercetin flavonoids levels increased during maturation (Chapter 4).

Kaempferol and quercetin flavonoids have similar UV-B absorbing properties but differ in anti-oxidation properties (Chapter 2; Wilson *et al.* 1998). It is supposed that quercetin flavonoids have another UV-B protecting function besides screening such as scavenging ROS, which are produced in enhanced amounts in response to UV-B. It has been observed in more plant species that especially flavonoids with an additional hydroxyl group in the B ring are UV-B induced (Bornman *et al.* 1997; Markham *et al.* 1998b; Mazza *et al.* 2000; Hofmann *et al.* 2000).

The less UV-B sensitive cv. Minica had a higher level of UV-B induced flavonoids (quercetin glycosides) than the more sensitive cv. Pistache. The concentration of constitutive flavonoids (kaempferol glycosides) was higher in the UV-B sensitive cv. Pistache. Also in several populations of *Trifolium repens* UV-B tolerance was related to especially quercetin flavonoids (Hofmann *et al.* 2000). Therefore, the amount of quercetin could be an indicator for UV-B sensitivity, which determines the UV-B induced attenuance (Chapter 4).

Localisation of UV-B absorbing compounds in a heterogeneous leaf system.

The third factor, which influences the UV attenuance on leaf tissue level, is the localisation of the flavonoids (see figure 1B). The attenuance of UV radiation is not homogeneously distributed over the leaf surface because of the heterogeneous localisation of flavonoids (Chapter 5; Day *et al.* 1993).

In some subcellular sites in the epidermal layer UV-B absorbing compounds are absent or less concentrated (e.g. stomatal pores and periclinal cell walls). These sites form gaps in the UV screen and reduce the screening effectiveness in spite of the spots with higher quercetin concentrations such as guard cells (Figure 1C; Chapter 5; Day *et al.* 1993; Vogelmann 1994). Despite these gaps, the leaf phenolics provide a good UV-B filter, which absorbs 99.9 % of the UV-B radiation (Chapters 4 and 5). Constitutive phenolics such as kaempferol, small amounts of quercetin and hydroxycinnamic acids could be observed in all leaf layers of *V. faba* (Chapter 5; Bornman *et al.* 1997; Winter & Herrmann 1986).

However, the heterogeneous localisation with, in general, a flavonoid concentration gradient across the leaf from the adaxial to abaxial side enhances the UV-B screening effectiveness (Figure 1B). UV-B induced quercetin accumulation was restricted to the adaxial epidermal layer in *V. faba* (Chapter 5; Figure 1B). This flavonoid distribution seemed to be very effective because the UV-B light is filtered out before it reaches the mesophyll tissues (Chapter 5; Bornman *et al.* 1997).

The question why flavonoids occur at specific sites in (UV-B irradiated) leaves like guard cells and epidermal cells (see figure 1C, right side) and to a lower extent in the spongy parenchyma (see figure 1C, left side) can be approached in several ways. First, the flavonoid synthesis can be triggered by exogenous stimuli like UV-B radiation. This seems to occur for the UV-B induced quercetin in the adaxial epidermis, which reflects the exposure to UV-B radiation. The abaxial epidermis, which was less exposed to UV-B radiation, indeed contained lower amounts of UV-B induced quercetin (Chapter 5). However, it is not likely that quercetin in the mesophyll is absent for this reason. Also the

mesophyll is exposed to small traces of UV-B radiation, which could trigger the quercetin pathway.

Second, flavonoids synthesis can be controlled endogenously (constitutive flavonoids). For constitutive kaempferol flavonoids, synthesis is part of a differentiation program (Winkel-Shirley 2001). In the process of evolution, the synthesis and accumulation of flavonoids have been proven useful for several functions such as UV-B protection by providing a UV screen (Cooper-Driver & Bhattacharya 1998; Rozema *et al.* 1997; Winkel-Shirley 2001).

Attenuance of UV-B radiation within the cells with microscreens (Figure 1C).

In addition to heterogeneous flavonoid localisation within the tissues, the flavonoids in *V. faba* leaves seemed to be partly sequestered at the subcellular level (Figure 1C; Chapter 5; Beerhues *et al. 1988*). Constitutive mesophyll flavonoids are probably less effective as general UV-B screen for the photosynthetic tissues. Instead of acting as a general UV-B screen, these constitutive flavonoids protect cells by forming microscreens (i.e. in regions with higher fluorescence) around specific organelles that are targets for UV-B radiation like the nucleus and chloroplasts (Figure 1C: spongy parenchyma; Chapter 5; Sheahan *et al.* 1998; Grandmaison & Ibrahim 1996). At these specific sites, it is a third trap for UV-B radiation that passed through the epidermal screen (Figure 1C).

The UV-B induced quercetin flavonoids in the adaxial epidermis accumulated mainly in vacuoles of epidermal cells (Figure 1C: adaxial epidermis). It is not clear if also epidermal flavonoids act as a microscreen. Also the specific role of a concentrated localisation of quercetin in guard cells (Chapter 5) is still not clear. Probably, it also forms a microscreen around the nucleus and chloroplasts (Sheahan *et al.* 1998; Grandmaison & Ibrahim 1996). It is also possible that the guard cell quercetin is the chromophore of a UV-B receptor, which is involved in stomata aperture and differentiation (Eisinger *et al.* 2003).

Conclusions

The main aim of this thesis was to study growth responses and morphological and chemical adaptation mechanisms to harmful UV-B radiation. Two cultivars of *V. faba*, differing in UV-B sensitivity were used as a model system.

Constitutive defence mechanisms appeared to function very well; even in the sensitive cv. Pistache the negative effects of enhanced UV-B were limited. The increased growth in response to UV-B demonstrated that especially cv. Minica was well adapted to

enhanced UV-B radiation. The differences in UV-B response between the two cultivars Pistache and Minica were mainly morphological differences of the whole plant (branching) and the amounts of UV-B induced quercetin. This caused differences in growth. The UV attenuation and total flavonoid accumulation and localisation of UV-B exposed leaves were comparable in both cultivars. Thus, I conclude that attenuation of UV-B radiation is very important and takes place at various hierarchical levels in the plant (Figure 1).

First of all, the external UV-B exposure was reduced by adaptations in the plant architecture (Figure 1A). In response to UV-B, plants showed a more compact stature with adventitious branches and shorter internode distance and plant height. This plant architecture leads to a denser canopy through which less UV-B radiation penetrated. Moreover, extra adventitious shoots with leaves compensated for the reduced length of the main stem.

Secondly, leaf morphology, chemistry and localisation of flavonoids contributed to UV-B attenuation on leaf tissue level. Leaves became thicker and smaller in response to UV-B (Figure 1B). Beside leaf thickness, kaempferol flavonoids in all leaf layers, non-soluble phenolics and hydroxy cinnamic acids contributed to the constitutive UV attenuance. The UV-B induced attenuance was achieved by quercetin flavonoids in the adaxial epidermis. In general, a flavonoid concentration gradient occurred across *V. faba* leaves, with highest concentrations in the adaxial epidermis and lower concentrations in palisade parenchyma and much lower concentrations in spongy parenchyma. This distribution of flavonoids over the leaves contributed to an optimal filtering of UV-B radiation, which is supposed to be one of the most important functions of flavonoids in the leaves.

The third level of attenuance was at the cellular level (Figure 1C). Microscreens of flavonoids around nucleus and chloroplasts of palisade parenchyma provided additional UV-B protection. It was a third trap for UV-B radiation, which had penetrated through the epidermal layers and which could damage the targets such as DNA and chloroplasts.

Among terrestrial flowering plants, legumes are generally considered to be relatively sensitive to enhanced solar UV-B radiation. However, the data of this thesis show that even UV-B sensitive plants such as *V. faba* are equipped with a multilevel UV attenuance system to deal with enhanced UV-B radiation. So, sensitivity to solar UV-B appears to be a rare phenomenon among terrestrial plant groups and research is needed to understand how well evolutionary older and younger plant groups are adapted to solar UV-B.

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Multilevel UV-B attenuance: the canopy structure is the first barrier for UV-B radiation. (*Vicia faba)*.

Summary

Multilevel UV-B Attenuance

Morphological and Chemical Adaptations of *Vicia faba* to Ultraviolet-B Radiation.

Multilevel UV-B Attenuance

Morphological and Chemical Adaptations of *Vicia faba* to Ultraviolet-B Radiation.

Due to anthropogenic reduction of stratospheric ozone, levels of solar UV-B radiation (280-315 nm) have been increasing on earth during the last three decades. This UV-B radiation is potentially harmful to all life on earth because of its energising wavelengths, which can cause photodestruction of biomolecules. In plants, DNA, membranes, and the photosynthesis apparatus are special targets for UV-B radiation. Molecular effects can result in reduced plant growth, bronzing and curling of leaves. However, plants possess, to a certain extent, the ability to cope with UV-B radiation. UV-B radiation can induce photomorphogenic effects such as thicker and smaller leaves, increased branching and induction of UV-B absorbing compounds (e.g. flavonoids), which contribute to UV-B protection. Also DNA repair mechanisms exist in plants. The balance between the damage, repair and adaptation processes, which are dependent on UV-B dose and other environmental factors such as PAR (Photosynthetically Active Radiation) level, determines the overall plant performance (e.g. plant growth) after enhanced UV-B exposure. Moreover, different plant species, and even different cultivars within one species, respond differently to enhanced UV-B radiation depending on the sensitivity and adaptation ability to UV-B radiation. This takes place at three levels: the whole plant, leaf tissue and cellular level.

The main aim of this thesis is to study the effects and adaptations to enhanced UV-B radiation. The focus is on adaptation to UV-B radiation on whole plant and leaf tissue level by attenuation of UV-B radiation. For this purpose, two cultivars, cv. Minica and cv. Pistache of *Vicia faba* (Faba bean) were selected, differing in UV-B growth response. This selection experiment is reported in **Chapter 1**. Despite an application of high levels of UV-B radiation (UV-B_{BE} 12 kJ m^{-2} d^{-1}, reflecting about 35% stratospheric ozone depletion), growth reduction is only observed in cv. Pistache, but not in the other four tested cultivars such as the most tolerant cv. Minica, which even demonstrates increased growth. Therefore, I have concluded that cv. Minica is more tolerant than cv. Pistache.

Secondary metabolites, especially flavonoids and hydroxycinnamic acids, are crucial in UV-B protection by absorption of UV-B radiation and are reviewed in **Chapter 2**. These compounds are synthesised in the phenylpropanoid and shikimate pathway. Some products of these pathways are induced by UV-B radiation, for example flavonoids with an additional hydroxyl group in the B ring (e.g. quercetin). Only a few examples of UV-B induced phenolic acids have been found. In addition to the UV-B dose, other factors

affect the quantity and quality of phenolics: plant species, cultivar, phenolic localisation in plant organs and tissues, and environmental conditions such as herbivory. The ecological and physiological implications of enhanced levels of flavonoids and hydroxycinnamic acids are also discussed in this chapter. For instance, an interaction between auxin and flavonoids with consequences for plant growth is hypothesised. Flavonoids and hydroxycinnamic acids also have other functions: as antioxidant agents, flower colours, and signalling molecules for *Rhizobium* interaction in Leguminosae.

Greenhouse experiments were used for the investigation of adaptation mechanisms to UV-B in plants described in this thesis, despite the more realistic light conditions in field experiments. Negative UV-B effects on terrestrial plants can be overestimated in growth chamber and greenhouse experiments due to lower PAR levels in these indoor environments when compared with field situation. Therefore, **Chapter 3** reports on the response of *V. faba* to enhanced UV-B radiation under low and near-ambient PAR levels in the greenhouse. The growth of cv. Minica of *V. faba* is stimulated in response to UV-B at both low and near-ambient PAR levels. Increased branching and number of leaves cause the positive UV-B response of cv. Minica. Concomitant with the increased branching, the biomass of the adventitious shoots also increases. The adventitious branches in cv. Minica also bear leaves which contribute to photosynthesis. In this way, the UV-B induced growth of the adventitious shoots compensate for the growth reduction of the main shoot in response to UV-B.

The appearance of the near-ambient PAR and UV-B exposed plants resembles plants with a loss in apical dominance (e.g. increased branching, reduced plant height and internode distance and reduced SLA [Specific Leaf Area]). Other similarities between UV-B and ambient PAR exposed plants are reduced leaf area and increased content of UV-B absorbing pigments in the leaves. This change in plant architecture and leaf morphology is advantageous in an enhanced UV-B environment. It contributes to a better attenuation of UV-B radiation on plant level. Less UV-B radiation penetrates the canopy and reaches the leaf tissues, so that damage to the photosynthetic mesophyll is reduced.

I conclude that the lower PAR level in the greenhouse and the unnatural light quality of the greenhouse environment in comparison to field situations can have subtle effects on plant morphology and plant chemistry, which, in time, can have an indirect effect on the UV-B sensitivity of the whole plant. However, the more controlled circumstances in the greenhouse make it easier to assign effects to the UV-B treatment and not to other environmental conditions.

As shown in the experiments described in Chapter 3, damaging and adaptive processes are interwoven. In spite of the positive growth response to UV-B on whole plant level, it is possible that the individual leaves of cv. Minica are sensitive to UV-B and are thus less productive in response to UV-B. Therefore, in **Chapter 4,** I have summarized my investigations into the attenuation of UV-B light by individual leaves as adaptation to UV-B. UV-B damage in the leaves is limited by UV-B attenuation on leaf tissue level so that it cannot reach potential targets (e.g. photosynthetic tissues and DNA). The effectiveness of the UV screen in the leaves depends on: 1) the thickness of this UV screen (leaf morphology), 2) the type and concentration of UV-B absorbing pigments (leaf chemistry) and 3) the localisation of the UV-B absorbing compounds such as flavonoids. The contribution of these factors to UV attenuance and UV-B induced attenuance are addressed in **Chapter 4 and 5**.

In **Chapter 4,** realistic *in vivo* UV attenuance measurements of intact *V. faba* (cv. Minica and cv. Pistache) leaves of different ages are presented. Moreover, I determined the contribution of flavonoid concentration, leaf thickness, non-soluble compounds and leaf developmental stage to *in vivo* UV attenuance. *In vivo* UV attenuance is coupled to flavonoid content by extracting soluble compounds from the leaf discs. By measuring the UV attenuance of the extracted leaf discs, I assessed the contribution of non-soluble compounds and leaf thickness to UV attenuance.

The transparency of leaves of *V. faba* for UV radiation was very low: 99.9% of the UV radiation was filtered out in non-exposed UV-B leaves (called constitutive attenuance). The attenuance in non-UV-B exposed leaves was eight times lower than in UV-B irradiated leaves. As a result, penetration of the harmful UV-B radiation appeared to be effectively reduced in leaves of both cultivars of *V. faba*. The UV-B induced attenuance (difference between attenuance of UV-B exposed and non-exposed leaves) was higher in cv. Minica than in cv. Pistache and built up during maturation of the leaves, whereas the constitutive UV attenuance was already present in young leaves.

Leaf thickness increased in response to UV-B, but correlations with *in vivo* attenuance were low. Moreover, the *in vivo* attenuance of extracted leaf discs was equal for *plus* and *minus* UV-B treatments. Thus, all UV-B induced attenuance disappeared after extraction from the leaf discs. I conclude that leaf thickness is not important for UV-B induced attenuance, however it is important as a constitutive UV screen, because only 16 % of the UV light passes through the extracted leaf discs. During maturation leaves became thicker, which made them less sensitive to UV-B radiation.

The total levels of flavonoids (quercetin plus kaempferol) in mature leaves were about 0.1 μmol cm^{-2} for *minus* UV-B and 0.2 μmol cm^{-2} for *plus* UV-B treated leaves. Total flavonoid amounts were equal in both cultivars, but cv. Minica had a higher level of UV-B

induced quercetin flavonoids than cv. Pistache. These quercetin concentrations increased during maturation concomitantly with the UV induced attenuance. A dilution of constitutive kaempferol was observed with maturation of the leaves because the leaves and cells were elongated. The concentration of kaempferol flavonoids, which were already synthesised in young leaves, was higher in cv. Pistache than in cv. Minica. In cv. Pistache, kaempferol concentrations were slightly enhanced in response to UV-A, but quercetin was not induced.

Leaf structure, hydroxycinnamic acids and non-soluble compounds contributed to the constitutive *in vivo* attenuance. Leaf flavonoid concentrations and *in vivo* UV attenuance were highly correlated in mature leaves. Therefore, I conclude that flavonoids contribute largely to the UV-B induced *in vivo* attenuance. Quercetin flavonoids are mainly responsible for the UV-B induced attenuance that is higher in the less sensitive cv. Minica. Therefore, the amount of quercetin, which determines the UV-B induced attenuance could be an indicator for UV-B sensitivity.

The localisation of flavonoids is reported in **chapter 5**. Because of the heterogeneous localisation, UV-B radiation transparency is not homogeneously distributed over the leaf surface. Sites where UV-B absorbing compounds were absent or less concentrated (e.g. stomatal pores and periclinal cell walls), formed gaps in the UV screen and reduced the screening effectiveness in spite of the spots with higher quercetin concentrations such as guard cells. However, the heterogeneous distribution of flavonoids from the adaxial (high flavonoids) to the abaxial leaf side (low flavonoids) implied a flavonoid concentration gradient. In both *plus* and *minus* UV-B treated *V. faba* leaves, the adaxial epidermal layer and the palisade parenchyma contained higher flavonoid concentrations than the spongy parenchyma. The flavonoid concentration in the abaxial epidermal layer was higher than in the spongy parenchyma but was lower than in the adaxial epidermis. As a result, the UV-B radiation is mainly filtered out before it reaches the UV-B sensitive mesophyll tissues.

UV-B induced quercetin accumulates mainly in the vacuoles and is restricted to the adaxial epidermal layer in *V. faba* and forms an important UV-B screen. Nevertheless, constitutive kaempferol and quercetin flavonoids occurring in the other leaf layers are also important. These compounds contribute to the UV attenuance on cellular level. However, these flavonoids may be less effective as a general UV-B screen for the photosynthetic tissues. Instead, these constitutive flavonoids may protect cells by forming microscreens around specific organelles that are targets for UV-B radiation like the nucleus and chloroplasts. The microscreens are regions with higher flavonoid concentration, which are visible as fluorescing spots in fluorescence microscope sections.

At these specific sites, they form a second trap for UV-B radiation passing the epidermal screen.

Thus flavonoids are localised at specific sites in (UV-B irradiated) leaf tissue like guard cells and epidermal cells and to a lesser extent in spongy parenchyma. Exogenous stimuli like UV-B radiation are responsible for the UV-B induced quercetin in the adaxial epidermis whereas the constitutive kaempferol flavonoids seem to be regulated endogenously as part of a differentiation program.

Chapter 6 addresses the general question of this thesis: what are the major adaptations of plants to UV-B so that severe UV-B damage is prevented? In this chapter, I present a conceptual model that shows how UV-B radiation is filtered out at three levels (whole plant, leaves and cells). Firstly, the external UV-B exposure is reduced by the changed plant architecture. In response to UV-B, a more compact plant growth occurs with adventitious branches and shorter plant height. This plant architecture will lead to a denser canopy through which less UV-B radiation penetrates. Secondly, thicker and smaller leaves will enhance UV-B attenuation at leaf tissue level. Moreover, a flavonoid concentration gradient in leaf tissues contributes to an optimal filtering of UV-B radiation. The third level of UV attenuance is found at the cellular level. Microscreens of flavonoids around the nucleus and chloroplasts of palisade parenchyma and guard cells provide additional UV-B protection. It forms a second trap for UV-B radiation which penetrates through the epidermal layers and which can potentially damage DNA and chloroplasts. Among terrestrial flowering plants, legumes are generally considered to be sensitive to enhanced solar UV-B radiation. However, this thesis shows that even UV-B sensitive plants such as *V. faba* are equipped with a multilevel UV attenuance system to deal with enhanced UV-B radiation.

UV-B straling: een peulenschilletje voor planten?

Samenvatting

UV-B absorptie op meerdere niveaus in de plant

Morfologische en chemische aanpassingen van *Vicia faba*
aan ultraviolet-B straling.

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UV-B absorptie op meerdere niveaus in de plant

Morfologische en chemische aanpassingen van *Vicia faba* aan ultraviolet-B straling.

Gedurende de laatste 30 jaar bereiken hogere UV-B stralingsdoses (280-315 nm) het aardoppervlak. Dit wordt veroorzaakt door de afname van de ozonconcentratie in hoge luchtlagen (de stratosfeer). Het evenwicht tussen ozonvormende en ozonafbrekende chemische reacties in de stratosfeer wordt door de mens beïnvloed, onder andere door de uitstoot van CFK's (Chloor Fluor Koolwaterstoffen). Hoewel de uitstoot van CFK's is afgenomen, is er nog geen herstel van de ozonlaag zichtbaar.

UV-B straling is in potentie schadelijk voor levende organismen door het hoge energieniveau van deze straling die biomoleculen kapot kan maken (fotodestructie). Bij planten kan dit tot geremde groei en zichtbare schade aan de bladeren leiden. Deze schade hoeft echter niet noodzakelijkerwijs op te treden omdat planten ook aanpassingen en herstel processen hebben, die ze bescherming bieden tegen UV-B straling (adaptatie). Voorbeelden van adaptatie aan hoge UV-B doses zijn dikkere en kleinere bladeren, meer vertakkingen en de aanmaak van UV-B absorberende pigmenten.

Hoe een plant uiteindelijk reageert op verhoogde UV-B straling, is afhankelijk van de UV-B dosis, de plantensoort, de cultivar en omgevingsfactoren zoals lichtspectrum en fotosyntheselichtniveau. De uiteindelijke plantengroei en ontwikkeling ("plant performance") worden bepaald door de balans tussen schadelijke processen aan DNA en fotosynthese enerzijds, en aanpassingen en herstelprocessen anderzijds. Bij lagere UV-B doses zullen de beschermende adaptatie en herstelprocessen de overhand hebben. Bij hogere doses domineren de schadelijke processen door fotodestructie van biomoleculen.

Het hoofddoel van dit proefschrift is het bestuderen van de effecten en adaptatie mechanismen aan verhoogde UV-B straling op plant- en bladweefselniveau. De Tuinboon (*Vicia faba*) is gebruikt als een modelgewas. In een eerste groei experiment zijn 5 cultivars getest op UV-B gevoeligheid, waarna twee cultivars geselecteerd werden, die verschillen in UV-B gevoeligheid. Ondanks de hoge biologisch effectieve UV-B stralingsniveaus ($UV\text{-}B_{BE}$ 12 kJ m^{-2} d^{-1}) die zijn gebruikt, is de zichtbare schade beperkt. Een groeireductie na UV-B blootstelling is alleen waargenomen bij cv. (cultivar) Pistache en niet bij de andere 4 geteste cultivars. Bij cv. Minica werd zelfs een groeistimulans waargenomen. Op basis van deze experimenten werd geconcludeerd dat cv. Minica toleranter is voor UV-B straling dan cv. Pistache (**Hoofdstuk 1**). Deze twee cultivars zijn

in de overige hoofdstukken gebruikt bij het experimentele onderzoek naar de effecten van, en adaptatie aan UV-B straling.

Een belangrijk UV-B beschermingsmechanisme bij planten is het vormen van een UV-B absorberend scherm in de bovenlaag van het blad (adaxiale epidermis). Dit scherm bestaat uit flavonoïden (pigmenten), die wel UV-B absorberen, maar niet het fotosyntheselicht. Daardoor kan de UV-B straling minder of geen schade toebrengen aan het UV-B gevoelige bladmesofyl, waar de fotosynthese plaatsvindt. Omdat het fotosyntheselicht niet geabsorbeerd wordt door de flavonoïden kan de fotosynthese wel ongehinderd plaatsvinden. Ook de hydroxykaneelzuren in het blad, hebben UV-B absorberende eigenschappen.

Hoofdstuk 2 geeft een literatuuroverzicht van deze UV-B absorberende stoffen in planten. Er wordt ingegaan op de biosynthese route van de flavonoïden: de fenylpropanoïde- en shikimaatroute. Ook wordt een overzicht gegeven van het voorkomen en de verhoogde productie van deze stoffen na UV-B blootstelling in diverse plantengroepen. Flavonoïden met een extra hydroxylgroep in de B-ring zoals quercetine, worden vaak gevormd onder invloed van UV-B. De productie van hydroxykaneelzuren wordt maar in een enkel geval verhoogd onder invloed van UV-B, terwijl dit voor de flavonoïden in de meeste gevallen wel gebeurt. Ook worden andere (omgevings)factoren besproken die het type flavonoïde en de gehaltes in de plant bepalen zoals bijvoorbeeld een verhoogd fotosyntheselichtniveau, de mate van vraat, het type weefsel en orgaan en de cultivar.

Functies van flavonoïden beperken zich niet tot het UV-B absorberende scherm. Het zijn ook anti-oxidanten die mee kunnen werken aan het voorkomen van UV-B schade door UV-B geïnduceerde radicalen weg te vangen. Andere functies van flavonoïden in de plant zijn bloemkleur en lokstof voor stikstofbindende wortelknolbacteriën (*Rhizobium*) bij vlinderbloemigen zoals de Tuinboon. Tenslotte wordt besproken wat de fysiologische en ecologische consequenties kunnen zijn van een verhoogd flavonoïde- of fenolniveau in de plant. Een verhoogd fenolniveau kan bijvoorbeeld de mate van vraat door insecten verminderen.

Ondanks de meer realistische lichtomstandigheden buiten in het veld zijn de experimenten, zoals beschreven in dit proefschrift, uitgevoerd in de kas. Negatieve UV-B effecten zijn in een kas of klimaatkamer meestal sterker dan buiten, omdat er in de kas meestal lagere lichtintensiteiten zijn. In **hoofdstuk 3** is onderzoek gedaan is naar de effecten van deze lagere lichtintensiteiten op de groeirespons bij verhoogde UV-B blootstelling.

De groei van *Vicia faba*, cv. Minica neemt echter ook in de kas, bij de lagere lichtintensiteiten, toe door UV-B straling (Hoofdstuk 3). Deze positieve groeirespons

wordt veroorzaakt door een groter aantal zijtakken en ook een groter aantal bladeren die door hun fotosynthese kunnen bijdragen aan een toenemende groei. De groei van de zijscheuten en dus de biomassaproductie van de zijscheuten compenseren voor de afnemende groei van de hoofdscheut.

Planten die zijn blootgesteld aan UV-B straling en/of verhoogd fotosyntheselicht-niveau hebben een vergelijkbare bouw en lijken op planten die hun apicale dominantie (deels) verloren hebben. Bij apicale dominantie zorgt de hoofdscheut ervoor dat de zijscheuten zich niet of minder ontwikkelen zodat voornamelijk de hoofdscheut doorgroeit. Planten met een verminderde apicale dominantie hebben dus meer en verder ontwikkelde zijscheuten, maar ook een kortere stengel en internodiën en een dikker blad. De compactere groeiwijze bij verlies van apicale dominantie kan beschouwd worden als een aanpassing aan UV-B straling op plantniveau. Andere overeenkomsten tussen de planten die opgekweekt zijn met UV-B straling of verhoogd fotosyntheselichtniveau zijn de kleinere bladeren en een toenemend gehalte aan UV-B absorberende pigmenten. Al deze veranderingen in de bouw en chemische samenstelling van de plant zijn functioneel in een omgeving met verhoogde UV-B-straling omdat er meer UV-B straling tegengehouden wordt in de bovenlaag van het bladerdek en er minder UV-B straling doordringt in het gewas waardoor er minder schade aan de fotosyntheseweefsel kan optreden.

Er kan geconcludeerd worden dat lagere lichtniveaus en onnatuurlijke lichtspectra in de kas slechts een subtiel effect hebben op plantmorfologie en -chemie en daardoor op de UV-B respons en UV-B gevoeligheid. Echter, door de gecontroleerde lichtomstandigheden in de kas kunnen effecten van UV-B straling beter onderscheiden worden van effecten die veroorzaakt door andere milieuomstandigheden.

Ondanks de positieve UV-B groeirespons op plantniveau is het mogelijk dat individuele bladeren van cv. Minica gevoelig zijn voor UV-B straling, en dat de biomassa productie per blad dus lager is. Om de UV-B gevoeligheid en respons op bladniveau te bepalen is de absorptie en transparantie van UV-B straling door individuele bladeren *in vivo* gemeten in **hoofdstuk 4**. De UV-B schade in de bladeren wordt beperkt doordat een groot deel van de UV-B straling geabsorbeerd wordt door flavonoïden en andere fenolen die een UV-B absorberend scherm vormen in het blad. De effectiviteit van dit scherm hangt af van: 1) de bladdikte, 2) de bladchemie: het type flavonoïde en de concentratie van deze UV-B absorberende stoffen en 3) de lokalisatie van deze flavonoïden in het blad. De bijdrage van deze factoren aan de UV-B absorptie en de door UV-B geïnduceerde extra UV-absorptie worden behandeld in hoofdstuk 4 en 5.

In **hoofdstuk 4** is de UV-absorptie bepaald van intacte bladeren (*in vivo* UV-absorptie) van cv. Minica en cv. Pistache. Ook is de invloed van de flavonoïden-

concentratie, de bladdikte en de bladleeftijd op de *in vivo* UV-absorptie bepaald. Hiervoor is de *in vivo* UV-absorptie gekoppeld aan de flavonoïdengehaltes. Dit is gedaan door de *in vivo* UV-absorptie te meten van verse bladschijfjes (bladponsjes). Vervolgens zijn de flavonoïden uit deze bladponsjes geëxtraheerd en is nogmaals de UV-absorptie door de geëxtraheerde bladponsjes bepaald. De UV-absorptie van de geëxtraheerde bladponsjes is een indicatie voor de bijdrage van niet-oplosbare fenolen en bladdikte aan de *in vivo* UV-absorptie. Het verschil in UV-absorptie tussen verse en geëxtraheerde bladponsjes geeft een indicatie van de bijdrage van de flavonoïden aan de UV-absorptie.

De transparantie van de bladeren voor UV-straling is erg laag: 99,9 % van de UV straling wordt weggefilterd, zelfs in niet aan UV-B blootgestelde bladeren. Dit wordt constitutieve UV-absorptie genoemd. De UV-absorptie in de UV-B blootgestelde planten is zelfs nog een factor 8 hoger. De schadelijke UV-B straling lijkt dus effectief gereduceerd te worden in bladeren van beide cultivars. De UV-B geïnduceerde UV-absorptie (absorptieverschil tussen wel en niet UV-B blootgestelde planten) is hoger in de minder gevoelige cv. Minica dan in de gevoelige cv. Pistache en wordt opgebouwd tijdens de ontwikkeling van het blad. De constitutieve UV-absorptie is al maximaal aanwezig in jonge bladeren.

Bladeren van aan UV-B blootgestelde planten zijn dikker, maar desondanks is de correlatie tussen bladdikte en *in vivo* UV-absorptie laag. Bovendien is de UV-absorptie van geëxtraheerde bladponsjes gelijk voor ponsjes afkomstig van wel en niet UV-B blootgestelde planten. Dus alle door UV-B geïnduceerde absorptie is verdwenen na extractie, zodat geconcludeerd kan worden dat de toename in bladdikte niet substantieel bijdraagt aan de UV-B geïnduceerde absorptie. Echter, de bladdikte en niet oplosbare stoffen zijn wel van belang voor de constitutieve UV-absorptie, want maar 16 % van de op het blad vallende UV-straling komt door de geëxtraheerde bladponsjes heen.

Het totale gehalte aan flavonoïden (quercetine en kaempferol) in volwassen bladeren is ongeveer 0,1 μmol cm^{-2} voor niet aan UV-B blootgestelde planten en 0,2 μmol cm^{-2} voor UV-B blootgestelde planten. De totale flavonoïdengehaltes zijn in beide cultivars (cv. Minica en cv. Pistache) gelijk maar de minder gevoelige cv. Minica heeft hogere UV-B geïnduceerde quercetine gehaltes dan de gevoeligere cv. Pistache. Cv. Pistache heeft daarentegen weer iets hogere (constitutieve) kaempferol concentraties.

De kaempferol gehaltes nemen af naarmate het blad zich ontwikkelt als gevolg van een verdunningseffect: alle kaempferol is in het jonge blad al aangemaakt. Dit verdunningseffect houdt in dat met de ontwikkeling van het blad, de cellen toenemen in volume, waardoor de concentraties van kaempferol in de cellen afnemen. Net als de kaempferol gehaltes is ook de constitutieve UV-absorptie maximaal in jonge bladeren en neemt deze af naarmate het blad veroudert. De gehaltes van het door UV-B

geïnduceerde quercetine daarentegen nemen toe met de ontwikkeling van het blad zodat ook de door UV-B geïnduceerde absorptie toeneemt. Het quercetinegehalte in het blad, en dus ook de UV-B geïnduceerde UV-absorptie, die hoger zijn in de minst UV-B gevoelige cv. Minica, zijn daarom mogelijk een goede indicator voor gevoeligheid voor UV-B.

De lokalisatie van de flavonoïden in het bladweefsel wordt besproken in **hoofdstuk 5**. De transparantie voor UV-B straling door het blad is niet homogeen verdeeld omdat de flavonoïden ook niet homogeen verdeeld zijn. Plaatsen waar de UV-B absorberende stoffen in lage concentraties aanwezig of helemaal afwezig zijn (bv. bij de celwanden en bij de openingen van de huidmondjes), vormen gaten in het UV-B scherm. Dit UV-B scherm functioneert daardoor minder goed, ondanks de plaatsen met hoge flavonoïden concentraties zoals de sluitcellen van huidmondjes.

De verdeling van flavonoïden van de bovenkant (adaxiale zijde) naar onderkant (abaxiale zijde) van het blad laat grofweg een concentratiegradiënt zien van hoog (bovenkant) naar laag (onderkant). Deze heterogene verdeling bevordert de efficiënte werking van het UV-scherm. In zowel UV-B blootgestelde als niet blootgestelde planten zijn de flavonoïden gehaltes het hoogst in de bovenepidermis (adaxiaal), waar het licht als eerste op valt. Het palissade parenchym (het deel van het mesofyl dat direct onder de bovenepidermis ligt) bevat ook hogere flavonoïdengehaltes dan het dieper in het blad liggende spons parenchym. De onderepidermis (abaxiaal) bevat weer hogere flavonoïden gehaltes dan het spons parenchym. De UV-B straling wordt dus weggefilterd voordat deze het mesofyl weefsel bereikt en daar schade zou kunnen aanrichten.

Het door UV-B geïnduceerde quercetine, dat de belangrijkste bijdrage levert aan door UV-B geïduceerde UV-absorptie accumuleert in de vacuoles van de adaxiale epidermis in *Vicia faba.* Niettemin, ook het constitutieve kaempferol in de andere bladlagen is van belang voor UV-B bescherming. Deze constitutieve flavonoïden zijn echter minder effectief in hun bijdrage aan het UV-B scherm in de epidermis voor bescherming van het fotosyntheseweefsel. Ze spelen een belangrijkere rol bij de vorming van zogenaamde microschermen. Rondom de gevoelige organellen zoals chloroplasten en de kern zijn deze stoffen geconcentreerd en beschermen zo de organellen tegen de UV-B straling die door de epidermale laag heen is gedrongen. De microscreens vormen dus een tweede beschermingsniveau in het blad.

Flavonoïden zijn dus gelokaliseerd op speciale plaatsen in het (aan UV-B blootgesteld) bladweefsel zoals sluitcellen van huidmondjes en andere adaxiale epidermiscellen. De flavonoïden zijn in mindere mate aanwezig in het spons parenchym. UV-B straling zorgt voor het door UV-B geïnduceerde quercetine in de adaxiale epidermis dat dus van buiten af (exogeen) gereguleerd wordt. Het constitutieve kaempferol

daarentegen, wordt endogeen gereguleerd als onderdeel van een differentiatie programma.

Hoofdstuk 6 gaat in op de algemene vraag van dit proefschrift: wat zijn de belangrijkste aanpassingen van planten aan UV-B straling zodat er geen ernstige UV-B schade ontstaat? In dit hoofdstuk wordt een conceptueel model uitgewerkt dat laat zien hoe UV-B straling op 3 niveaus (plant-, weefsel- en celniveau) wordt weggefilterd. Allereerst wordt de UV-B blootstelling van het gewas gereduceerd door een veranderde plant architectuur. Onder invloed van UV-B ontstaat er een compactere kortere plant met meer vertakkingen en kortere internodiën. Daardoor wordt het gewas dichter en kan UV-B straling moeilijker in het gewas doordringen.

Ten tweede leiden dikkere en kleinere bladeren tot minder last van UV-B straling. Ook de flavonoïdengradiënt in het blad zorgt voor een optimale UV-B absorptie.

Het derde niveau waarop verzwakking van UV-B straling optreedt is op celniveau. Microschermen van flavonoïden rond de kern en de chloroplasten van het palissade parenchym zorgen voor een extra filtering van de UV-B straling die door de abaxiale epidermis is heen gedrongen. Ze beschermen de extra UV-B gevoelige kern en chloroplasten.

De resultaten in dit proefschrift laten zien dat UV-B gevoelige planten zoals de Tuinboon (*Vicia faba*) op diverse niveaus zijn uitgerust met een systeem voor absorptie van UV-B-straling zodat ze goed aangepast zijn aan verhoogde UV-B straling.

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Barbara.

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Curriculum vitae

Barbara Meijkamp werd op 30 augustus 1969 geboren in Tilburg. Na het behalen van haar Atheneum-B diploma aan het St. Odulphuslyceum in Tilburg begon zij haar studie in Wageningen aan de Internationale Agrarische Hogeschool Larenstein. In het kader van haar studie Laboratoriumtechniek, afstudeerrichting Botanie deed zij stages bij de afdeling Landschapsecologie van de Universiteit Utrecht en afdeling Siergewassen van het CPRO-DLO instituut. Na haar HBO opleiding studeerde ze Biologie aan de Landbouw Universiteit Wageningen, waar ze zich specialiseerde in de plantenfysiologie en fytochemie. Tijdens haar afstudeervak bij de vakgroep Plantenfysiologie deed ze onderzoek naar de groei en anthrachinon productie van *Morinda citrifolia* celsuspensies.

Van 1995 tot 2000 werkte zij als Assistant in Opleiding bij de afdeling Plantenoecologie en later Systeemoecologie van de Vrije Universiteit (VU) Amsterdam. Zij bezocht diverse internationale congressen en symposia in binnen- en buitenland waar zij haar werk presenteerde. Het onderzoek dat in deze periode werd uitgevoerd staat in dit proefschrift beschreven.

Naast haar promotieonderzoek behaalde zij haar pedagogisch-didactische aantekening voor HBO-docenten. Van 2000 tot 2001 deed zij onderwijskundig onderzoek naar "good practice" bij ANW (studiehuisvak Algemene NatuurWetenschappen) bij het Instituut voor Didactiek en Onderwijspraktijk van de VU. Dit onderzoek resulteerde in een serie portretten van scholen waar "good practice" van ANW toegepast werd.

Vanaf 2001 is zij werkzaam als docent bij de Academie voor de Technologie van Gezondheid en Milieu (ATGM) van de Avans Hogeschool (Breda). Ze begeleidt (afstudeer)studenten (BSc) en ontwikkelt en verzorgt (Engelstalig) onderwijs voor Nederlandse en internationale studenten Milieukunde (Environmental Technology and Management). Haar vakgebieden liggen op het terrein van Ecologie, Natuur- en landschapsbeheer, Milieubiologie, Toxicologie en Duurzame landbouw.

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